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Elucidation of a bacteriophytochrome-regulated signal transduction pathway in Pseudomonas syringae that contributes to leaf colonization, virulence, and swarming motility

Regina S. McGrane
Iowa State University

Elucidation of a bacteriophytochrome-regulated signal transduction pathway in *Pseudomonas syringae* that contributes to leaf colonization, virulence, and swarming motility

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Regina S. McGrane

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Program of Study Committee:
Gwyn A. Beattie, Major Professor
Mark L. Gleason
David J. Hannapel
Leonor F. S. Leandro
Gustavo C. Macintosh

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DEDICATION

To my husband, who never stops believing in me, without his support this would not be possible. To my parents and grandparents who have always encouraged me in the pursuit of my hopes and dreams. Thank you all for your support during this long journey.

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ABSTRACT

Plant-associated bacteria encounter a range of stressful environmental conditions when colonizing leaf surfaces. To adapt to these harsh conditions bacteria sense and respond to environmental signals. Within the last two decades, photoreceptors that respond to specific wavelengths of light through associated chromophores have been discovered with increasing frequency in non-photosynthetic bacteria, including those associated with plants. Their presence suggests that fluctuations in light may serve as a cue to regulate bacterial adaptations. The foliar plant pathogen *Pseudomonas syringae* is unusual among heterotrophic bacteria because it encodes three photoreceptors, two red- and/or far-red light-sensing bacteriophytochromes and a blue light-sensing LOV protein. Here we evaluated the physiological roles of these photoreceptors, their mechanisms of regulation, and their impacts on plant colonization. This work provides the first evidence in bacteria for an integrated signaling network composed of both a LOV protein and a phytochrome, and shows that the bacteriophytochrome, BphP1, and LOV control swarming motility. BphP1 represses swarming motility in response to red and far-red light, whereas LOV attenuates BphP1-mediated repression. Moreover, this is the first bacteriophytochrome shown to have blue-light sensing capabilities, and these occur independently of red-light sensing. Furthermore, this work identifies a role for a bacteriophytochrome in plant colonization for the first time and demonstrates that this bacteriophytochrome, BphP1, promotes survival during the initial stages of leaf colonization and negatively regulates colonization later on. BphP1-mediated regulation of swarming motility is associated with the ability of *P. syringae* to move from soil to seeds and contributes to lesion development. This work further elucidates the mechanism of BphP1-mediated regulation and demonstrates that BphP1 and a regulator we designate Lsr repress swarming motility in response

to red light by controlling the transition from a sessile to motile lifestyle. Additionally, an acyl-homoserine lactone molecule functions as a downstream component in this signal transduction pathway. This work also provides evidence for light-mediated regulation of the type IV pilus and demonstrates for the first time a role for type IV pili in the swarming motility of *P. syringae* pv. syringae. Furthermore, the global regulator AlgU is shown to negatively regulate swarming independent of alginate production, which itself enhances swarming motility. Finally, the work documents an unusual interaction between *P. syringae* colonies that manifests as induced movement away from colonies producing the repellent 3-(3-hydroxyalkanoyloxy) alkanoic acid by strain derivatives that otherwise appear non-motile.

GENERAL INTRODUCTION

The roles of photoreceptors in plants are well understood; however, until recently the roles of photoreceptors in prokaryotes have been largely unstudied. Photoreceptors are commonly encoded by plant-associated bacteria, suggesting that light sensing may be an important adaptation, particularly when colonizing the phyllosphere. The phyllosphere, specifically the leaf surface, is exposed to a variety of fluctuating environmental conditions that may correlate with changing qualities and quantities of light. Specifically, increased temperatures and low water availability likely occur during maximum light exposure. The foliar pathogen *Pseudomonas syringae* is unique among heterotrophic bacteria because it encodes genes for three distinct photoreceptors, suggesting that light sensing may be an important adaptation for its survival. *P. syringae* encodes two red- and/or far-red-light sensing bacteriophytochromes: BphP1, which encodes a C-terminal HisKA histidine kinase domain, and BphP2, which encodes tandem HisKA-HWE histidine kinase domains, and a blue light-sensing LOV protein. Light sensing may serve as a cue to initiate mechanisms of adaptation that allow *P. syringae* to maintain large epiphytic leaf populations, which are precursors for disease symptoms. The goal of this work is to identify the physiological processes regulated by light through *P. syringae* encoded photoreceptors. We hypothesize that integration of signaling from specific wavelengths may allow *P. syringae* to evade light-regulated plant defenses. To elucidate the signal transduction pathway that controls light-mediated physiological processes we investigate proteins that interact with and/or regulate swarming downstream of BphP1. Additionally, we evaluate the role of BphP1 and LOV in plant colonization and virulence and characterize the influence of light-mediated regulation of swarming in movement in soil.

Motility is known to contribute to the ability of *P. syringae* to survive on and colonize leaves, and genes associated with swarming are highly induced in *P. syringae* cells associated with the plant. To better understand the regulation and mechanism of swarming motility we evaluate the relative contributions of flagella and type IV pili, biosurfactants, alginate, and the global regulators AlgU and GacA in swarming and evaluate their role in light-mediated regulation of swarming. Furthermore, we characterize a movement that we designate translocation exhibited by mutants deficient in swarming motility. Collectively, this work evaluates the physiological role for photoreceptors in *P. syringae* swarming motility and plant colonization in addition to investigating the mechanisms and regulation of swarming motility.

Dissertation organization

This dissertation is divided into five chapters. Chapter 1 provides a review of the relevant literature relating to the *Pseudomonas syringae* lifecycle, mechanisms of light sensing, and the mechanisms and roles of motility in *P. syringae*. Chapter 2 demonstrates the role of a bacteriophytochrome and LOV in integrated regulation of swarming motility. Chapter 3 provides evidence for bacteriophytochrome regulation of plant colonization and virulence in addition to further elucidating the signal transduction pathway of light-mediated swarming regulation. Chapter 4 characterizes the role of the type IV pili, biosurfactants, alginate, and the global regulators AlgU and GacA in swarming motility in addition to investigating a new type of surface motility we designate translocation. Chapter 5 describes my conclusions and possible avenues of future research.

CHAPTER 1. LITERATURE REVIEW

Pseudomonas syringae as a plant pathogen and model for plant-bacterial interactions

Pseudomonas syringae is a plant pathogen that primarily causes foliar disease on a variety of economically important crops; symptoms include necrotic lesions, which form following invasion of plant tissues (1). *P. syringae* can be grouped into over 50 pathovars that vary in host specificity (1). Whereas most pathovars cause foliar diseases, some cause alternative diseases including spots and specks on fruit, blights on shoots and flowers, stalk rot, galls, and cankers on tree limbs and trunks. *P. syringae* strains have been studied as models for the type III secretion system (T3SS), which is important for virulence in most gamma-proteobacterial pathogens of animals and plants. This has allowed *P. syringae* mechanisms of pathogenicity to be well understood among bacterial plant pathogens. Three strains have been fully sequenced and widely studied: *P. syringae* pv. tomato DC3000, *P. syringae* pv. syringae B728a, and *P. syringae* pv. phaseolicola 1448A (2-4); each of these strains represents a unique monophyletic group. Comparative genomics have been applied in multiple studies in the last decade to understand strain differences in genes involved in epiphytic fitness, effector secretion pathways, and virulence regulation (3-5).

P. syringae pv. syringae B728a causes brown spot on common bean and is able to maintain high epiphytic populations (6), which can reach densities of 10^7 cells per gram of leaf tissue on common bean leaves (7, 8). High epiphytic populations serve as reservoirs for disease outbreaks (9), which often occur following intense rains that trigger rapid population increases (10). Above ground plant surfaces are collectively referred to as the phyllosphere and are exposed to a variety of fluctuations in environmental conditions including temperature, humidity,

and light (6). During colonization of leaves, *P. syringae* is exposed to environmental stresses including ultraviolet radiation, oxidative and osmotic stress, and nutrient-limiting conditions (5).

Much of the work studying *P. syringae* has focused on the factors that contribute to its ability to cause disease. *P. syringae* pv. syringae B728a produces two phytotoxins that contribute to virulence, syringomycin and syringopeptin. These are amphipathic toxins that can form pores in plant membranes resulting in flow of nutrients from the plant cell (11). Additional virulence traits include translocation of effector proteins, which repress plant immunity and basal defenses, into host cells via the T3SS (9). Collectively, the effectors and proteins involved in biosynthesis of the T3SS are referred to as the Hrp (hypersensitive reaction and pathogenicity) system and many of these are encoded in the *hrp/hrc* cluster (12). Loss of the T3SS severely reduces pathogenicity, while each effector is predicted to have minor, but additive roles in pathogenicity (13). Effectors often interact with corresponding protective chaperones which prevent degradation and contribute to translocation (14).

*P. syringa*e utilizes a variety of mechanisms to adapt to environmental stresses encountered on leaves. Phyllosphere bacteria utilize motility and chemotaxis for survival because of the heterogeneous distribution of nutrients and water on leaves (15). In response to water-limiting conditions, *P. syringae* synthesizes alginate, which functions to promote water absorption (16), and the compatible solutes NAGGN and trehalose (17, 18), which balance the osmotic difference between the bacteria and the environment. The quaternary ammonia compounds choline (19, 20), glycine betaine, and carnitine (21) function as osmoprotectants and are accumulated in *P. syringae* cells through uptake from the environment by various transporters. Additionally, *P. syringae* forms aggregates near glandular trichomes and along veins, which are predicted to be sites where water and nutrients are freely available, and these

aggregates provide protection from desiccation (15). In response to ultraviolet radiation *P. syringae* expresses *rulAB,* which encode a protein involved in mutagenic repair; this repair system helps prevent cell death due to radiation-induced mutations (22, 23). Although the mechanisms by which *P. syringae* tolerates exposure to water limitation and ultraviolet light are understood, the impact and responses to visible light remain poorly understood.

Light sensing

The ability to sense changes in light can be found in every kingdom of life (24). Changes in light cue organisms to the time of day (25) and their position relative to the sun within aquatic and terrestrial habitats (24). In plants, red-light sensing induces seed germination, leaf formation, and flowering time (26), while blue light increases photosynthetic potential (27, 28). Ultraviolet and blue light can be detrimental to cells due to their contribution to DNA damage (29), photoexcitation promoting the generation of reactive oxygen species, and photosensitization of porphyrins and flavins (30).

In eukaryotes and prokaryotes light is perceived through photoreceptor proteins that are grouped into six families based on the structure of their associated light-absorbing chromophore (31). Each family encodes a photosensory domain that interacts with a light-absorbing chromophores, which change conformation in response to specific wavelengths of light and shift the associated protein to a signaling state (31). The six families consist of red and/or far-red light-absorbing phytochromes (32), and five proteins that respond to blue light: light, oxygen, and voltage (LOV) domain-containing proteins (33), photoactive yellow proteins (PYP) (34, 35), blue light-sensing FAD (BLUF) proteins (36), cryptochromes (37), and rhodopsins, which can also function as green light sensors (38). These photosensory proteins often consist of an N-terminal photosensory domain, which interacts with a chromophore and a C-terminal output

domain. In this work, we evaluate the role of light sensing in the lifecycle of *P. syringae* pv. syringae B728a.

Bacteriophytochromes

Bacteriophytochromes are among the red and/or far-red light-absorbing phytochromes (39). Bacteriophytochromes were first discovered in heterotrophic bacteria by screening sequenced genomes for genes that encode regions with similarity to the chromophore-binding domains of Cph1, RcaE, and Ppr from cyanobacteria (39). Today they are known to be the most common light-sensing protein in bacteria and have been discovered in 17% of sequenced bacterial genomes (40). The N-terminal photosensory domain of bacteriophytochromes consists of a phytochrome domain (PHY), PAS and GAF domains, and chromophore binding domain (CBD) (41). The CBD interacts with the chromophore biliverdin at conserved histidine and cysteine residues through a Schiff-base-type linkage (42). In response to excitation by red and/or far-red light, covalently bound biliverdin is interconverted by isomerization of the 15-16 double bond of the bilin chromophore (41). The mechanism by which the signal is transmitted following interconversion is not well understood; however, interconversion initiates a switch from the Pr form, which absorbs red light, to the Pfr form, which absorbs far-red light (42). Following the conformational change, the C-terminal output domain enters a signaling state (42).

P. syringae encodes two bacteriophytochromes, BphP1 and BphP2 (42). Both consist of an N-terminal photosensory domain that interacts with biliverdin and a C-terminal histidine kinase domain (42). BphP1 and BphP2 are predicted to form homodimers where one member of the pair binds ATP and the other provides the histidine phosphoacceptor (43). Each has a HisKA histidine kinase domain that consists of six motifs: an H-box which includes the conserved histidine that is the site of phosphorylation, N, G1, G2 and G3 boxes involved in defining the

nucleotide-binding cleft, and an F-box (43). BphP2 has a second unique HWE histidine kinase domain that lacks an F-box; this unusual histidine kinase domain was first discovered in a bacteriophytochrome in *A. tumefaciens* (43). It is noteworthy that *P. syringae* BphP2 is the only bacteriophytochrome that has this unique tandem HisKA-HWE domain structure. The gene that encodes BphP1 is upstream of *bphO,* which encodes a heme oxygenase (42). This heme oxygenase converts heme into the chromophore biliverdin (42) and is critical for proper BphP1 folding during translation and the subsequent incorporation of biliverdin into BphP1 (44). BphP1 autophosphorylates in the Pfr form in response to red light when in the presence of biliverdin (42). The biochemical properties of BphP2 have not been evaluated due to resistance to purification (44).

The biochemical properties of bacteriophytochromes have been well studied, but the physiological processes they regulate have not. In *Bradyrhizobium* ORS278 and *Rhodopseudomonas palustris* bacteriophytochromes directly initiate synthesis of the photosynthetic apparatus in response to far-red light; however, this regulation is not conserved among distantly related anoxygenic bacteria (45, 46). *Rhodopseudomonas palustris* bacteriophytochrome also inhibits respiratory activity in response to far-red light, suggesting that it allows the bacteria to fine tune its bioenergetic processes in response to changing light conditions (47). Among heterotrophic bacteria, a bacteriophytochrome induces production of the pigment deinoxanthin in *Deinococcus radiodurans* (39), while a bacteriophytochrome is regulated by the Las quorum sensing network in *Pseudomonas aeruginosa* (48). To date, the role of bacteriophytochromes in bacteria that colonize or cause disease in plants has not been examined. In this work, we investigate the physiological responses regulated by

bacteriophytochromes in *P. syringae* pv. syringae B728a and evaluate their implications in plant colonization and virulence.

LOV proteins

LOV domains were first identified in proteins in *Arabidopsis thaliana* (49) and have since been classified as a type of PAS domain (50). A LOV domain-containing protein encoded by *Bacillus subtilis* was the first prokaryotic LOV protein discovered (51), and LOV proteins have since been predicted to be present in 13% of bacterial species (40, 52). Flavins function as chromophores that interact with LOV domains at conserved cysteine residues (53). Upon excitation by blue light, the flavin forms an adduct (53) activating the C-terminal output domain. These output domains range from histidine kinases to sulfate transporter anti-σ antagonists, helix-turn-helix and GGEF-EAL motifs, globin, CheB or CheR domains, and cyclase 4 domains (54). Histidine kinase domains are the most common and are found in 50% of bacterial LOV proteins (54). The flavin adduct forms between a carbon of the flavin isoalloxazine ring and a conserved cysteine residue (55). Following this formation, the adduct slowly decays to a ground state with a half-life from minutes to hours (56) depending on the pH and salt concentration (54).

The LOV protein encoded by *P. syringae* interacts with a flavin mononucleotide and the adduct decays with a half-life of 22 min (57). *P. syringae* LOV encodes a histidine kinase domain in addition to a C-terminal response regulator domain (58). This LOV-HK-REC hybrid protein structure is almost exclusively found in bacterial plant pathogens (59). *P. syringae* LOV autophosphorylates in response to blue light, and when expressed as two truncated protein fragments on separate plasmids, the autophosphorylated histidine kinase domain transfers its phosphoryl group to the response regulator (58).

LOV proteins have been studied in a diverse set of heterotrophic bacteria, and while a trend in physiological function is beginning to emerge, many questions remain unanswered. Among the organisms studied LOV proteins seem to regulate phenotypes associated with either biofilm formation and attachment or motility, prompting the speculation that LOV regulates a switch between a single motile cell and a multicellular sessile lifestyle, that is, a biofilm (40). In line with this hypothesis is evidence that LOV proteins regulate attachment- and biofilm-associated behaviors in *Xanthomonas axonopodis* pv. citri (59), *Rhizobium leguminosarum* (60), and *Caulobacter crescentus* (55). Additionally, in both *P. syringae* pv. tomato DC3000 (61) and *Xanthomonas axonopodis* pv. citri (59), a LOV protein regulates motility. A LOV protein is also associated with disease symptoms in both *P. syringae* pv. tomato DC3000 (61) and *Xanthomonas axonopodis* pv. citri (59) along with proliferation in macrophages in *Brucella abortus* (58). There also seems to be some conservation in the role of LOV proteins in regulating bacterial stress responses. In *Caulobacter crescentus* a LOV protein coordinates its regulation of stress response with the two-component regulator PhyK-PhyR (62) and the LOV protein encoded by *Erythrobacter litoralis* HTCC2594 interacted with two PhyR-like response regulators *in vitro* (63). Additionally, *P. syringae* pv. tomato DC3000 LOV regulates resistance to oxidative stress (61) and expression of the genes for the sigma factors RpoS, RpoN, and HrpL (64). While a picture of the function of LOV proteins in heterotrophic bacteria is beginning to emerge, there are still many unanswered questions, including the pathways through which LOV proteins regulate biofilm, motility, and stress-related phenotypes. The goal of this study is to characterize the role of LOV in *P. syringae* pv. syringae B728a motility and determine its function in plant disease.

Light-mediated pathogen resistance in plants

Whereas the contribution of light to plant resistance to pathogens has been known for some time, the role of circadian rhythms in this resistance has been examined only recently with increasing frequency in *Arabidopsis thaliana* (65). Over 89% of the *A. thaliana* genome is expressed in an oscillating manner that parallels diurnal cycles (66); this includes the flagellin receptor FLS2, which is required for PAMP-triggered immunity (PTI) (67). The peak expression of FLS2 occurs in the morning, which is the time of day when the stomata are beginning to open and the bacterial populations are at their highest (67, 68). Loss of circadian-regulated genes results in differences in the susceptibility of plants depending on the time of inoculation with a pathogen (67). Additionally, production of salicylic acid (SA), which is involved in plant innate immunity, and activation of downstream SA-mediated defense responses are both light-dependent processes (65). Specifically, light is required for the induction of the hypersensitive response, based on the observation that plants inoculated with incompatible pathogens showed reduced lesion formation when kept in the dark (69). Systemic acquired resistance (SAR) is also light specific; plants inoculated with avirulent strains in the light are resistant to subsequent infection by pathogens, whereas those inoculated in the dark do not exhibit SAR (69). Collectively, plants use light signaling as a cue to prepare and respond to the presence of nonpathogenic and pathogenic bacteria.

Bacterial motility

Bacteria utilize diverse mechanisms to move and colonize habitats. Among Pseudomonads the three types of characterized motility are twitching, swimming, and swarming. Twitching motility occurs when bacteria are associated with a moderately viscous surface comparable to 1% agar (70). Cells that twitch can act as individuals or in groups composed of

rafts of aligned cells (70). Twitching requires the production of the type IV pilus, which attaches to a surface and then pulls the cell forward by retraction (71). Swimming motility is often referred to as flagellar motility as it requires a single flagella and is a single cell behavior that occurs in liquid (72). Swarming motility is a collective, group movement across a semi-solid surface that requires flagella and a thin film of water (73). Many organisms require some type of wetting agent, through the production of either biosurfactants that reduce surface tension or compounds such as polysaccharides that attract water (74). *Pseudomonas aeruginosa* has a fourth type of motility referred to as sliding that occurs independent of flagella and type IV pili; sliding is promoted by the presence of rhamnolipid surfactant (75). A variety of pathways and environmental cues regulate each type of motility. In this work, we evaluate the role of light sensing in regulating swarming motility.

Hierarchical expression of the flagellar components

Flagellar assembly requires a large amount of energy, therefore expression of flagellar biosynthesis genes are tightly regulated to ensure that each component is produced in the correct order (76). This regulation has been extensively studied in *Pseudomonas aeruginosa* and is likely conserved in other species, although this has not yet been confirmed. The master regulator FleQ controls expression of flagellar biosynthesis in a four-tiered cascade that includes multiple targets for regulation (77). Each of the four tiers corresponds to a class of genes. FleQ is the only Class I protein and it initiates expression of the Class II genes *flhF*, *fliEFG*, and *fleN* (78). FlhF determines the placement of the new flagellum, whereas FliEFG forms the rotor, switch, basal body and export apparatus (78). FleN then represses *fleQ* to ensure the cell produces only one flagellum (78). This repression of *fleQ* initiates the FleS/FleR two-component system, which activates expression of the Class III genes and completion of the hook-basal body structure (78).

The sigma factor FlgM is then exported through the completed hook-basal body, which releases the cognate sigma factor FliA (78). FliA promotes the expression of the Class IV genes including *fliC* and *fleL*, which encode proteins for the flagellum monomer and a regulator of flagellum length, respectively (78).

In *P. syringae* pv. syringae B728a insertional mutations in *fleQ*, *flgC*, and *fliC* all resulted in reduced swarming motility (79). This regulatory cascade of flagella biosynthesis is temperature regulated through FlgM, which controls FliA regulation of *fliC*, resulting in reduced flagellin production above 28°C (80). Loss of a functional flagellum via mutagenesis severely reduces virulence in multiple *P. syringae* pathovars (81, 82) and reduces the ability of cells to move to sites that are protected from environmental stresses (83). Additionally, genes associated with flagellar biosynthesis were induced in cells colonizing epiphytic (leaf surface) sites, but were not induced in cells colonizing apoplastic (intercellular) sites (8). Collectively, these results suggest that flagellar-mediated motility is important for *P. syringae* survival on leaves. Apart from its role in motility, the monomer subunit of flagella, flagellin, is an important cell surface feature designated as a microbe-associated molecular pattern (MAMP) that is recognized by plant receptors that are involved in inducing basal defenses against bacteria (16, 81, 84).

Flagellar modifications required for swarming motility

To adapt to the increased power required to traverse a semi-solid surface many organisms produce multiple flagella (85), with Pseudomonads producing two polar flagella rather than one (76). Pseudomonads encode two separate flagella stator complexes; these hold the rotor in place and provide the torque for flagellar rotation by transporting protons across the inner membrane (86, 87). In *P. aeruginosa* the two stator complexes, MotAB and MotCD, are functionally redundant in their role in swimming motility (86, 87). In contrast, only MotCD can provide the

necessary torque for swarming motility, suggesting that the flagellar motor is reconfigured in response to increasing surface resistance (86, 87). *P. syringae* has genes encoding both MotAB and MotCD complexes; however, the role of these complexes in swarming motility has not been evaluated.

Glycosylation of the flagellum subunit protein flagellin contributes to swarming motility in the *P. syringae* strains examined. In *P. syringae* pv. tabaci 6605, glycosylation is mediated by three glycosyltransferases, which attach six glycans to serine residues in the center of flagellin (88). Loss of any of the glycosyltransferases results in reduced swarming motility (88). In *P. syringae* pv. syringae B728a insertional mutants of the glycosyltransferases *fgt1* and *fgt2* also exhibit reduced swarming motility (79). The glycosyltransferases appear to have a feedback role in expression of late-stage flagellar biosynthesis genes; loss of *ftg2* resulted in upregulation of *fliC* but did not influence expression of early-stage genes like *fliE* or *flgB* (79). *P. syringae* pv. tomato DC3000 and *P. syringae* pv. phaseolicola 1448a also encode glycosyltransferases and their chemical structures are similar to those encoded by *P. syringae* pv. tabaci 6605, although their role in swarming has not been characterized (89). These posttranslational modifications are predicted to stabilize the flagella in environments with increased viscosity (90).

Biosurfactants

Biosurfactants are molecules synthesized by bacteria that reduce surface tension and act as lubricants. They are amphiphilic compounds containing an amino acid chain and a fatty acid tail (91), which allow them to interact with hydrophilic and hydrophobic surfaces, respectively. By reducing surface tension, biosurfactants increase the spread of water droplets, leading to reduced frictional forces so less torque is required to traverse semi-solid surfaces (85).

Biosurfactants are regulated by a range of physiological processes and environmental factors including temperature, pH, and salinity (91).

P. aeruginosa produces an amphiphilic glycolipid called rhamnolipid that is required for swarming motility and has been studied in detail (92). Dirhamnolipids promote bacterial motility and chemoattraction, whereas monorhamnolipids act as wetting agents (76). The precursor of rhamnolipid is 3-(3-hyroxyalkanoyloxy) alkanoic acids (HAA) (93) acts as a repellent and prevents intersection of tendrils in close proximity (76). Expression of the *rhlAB* operon, which encodes proteins required for the synthesis of rhamnolipid, is regulated by the sigma factor σ^s and the quorum regulators RhlR and LasR (76). The small RNAs RsmZ and RsmY, which are under control of the GacA/GacS two-component system, provide post-transcriptional regulation (94). *P. syringae* encodes *rhlA* which directs the synthesis of HAA (79); however, while it produces this rhamnolipid precursor it does not produce rhamnolipid, likely due to the lack of *rhlB* which stimulates monorhamnolipid production (76).

Syringafactins A-F are the primary surfactants contributing to swarming motility in *P. syringae* (95) whereas HAA contributes but has a minor role (79). Syringafactins A-F are six structurally related linear lipopeptides that are produced by a single gene cluster encoding a non-ribosomal peptide synthase (95). As distinctions have not yet been made between the function of each linear lipopeptide they are collectively referred to as syringafactin. Five genes are involved in the production of syringafactin; two nonribosomal peptide synthase genes, *syfA* and *syfB*, along with three additional genes *syfC*, *syfD*, and *syfR* (95). Loss of either *syfA* or *syfB* resulted in the complete loss of motility by DC3000 cells and reduced swarming by B728a cells (96). Syringafactin production is temperature regulated with complete repression of *syfA* occurring at 28°C (80). Syringafactin regulation by temperature is dependent on acyl-CoA dehydrogenase

(ACDH) and a nucleoside diphosphate hydrolase RppH; however, the mechanism of their role in regulating *syfA* expression is not understood (97). Iron also contributes to regulation with iron starvation and loss of the siderophore pyoverdine leading to an increase in syringafactin production, suggesting that swarming motility is an important mechanism utilized during iron scavenging (98). Syringafactin contributes to fitness on leaves under fluctuating humidity conditions in part due to its role in motility but also through its ability to increase the rate of nutrient diffusion across the cuticle (99).

The observation that loss of syringafactin production had different effects in DC3000 and B728a resulted in the discovery that B728a also produces HAA whereas DC3000 does not (79). The production of this second biosurfactant is coordinated with the expression of late-stage flagellar genes (79). Loss of early-stage flagellar gene expression causes reduced *rhlA* expression, whereas loss of late-stage flagellar gene expression causes increased *rhlA* expression; however, FleQ is not required for HAA production (79). These results suggest that HAA production is increased in early stages of flagella production and repressed when *fliC* is expressed (79). *rhlA* is also regulated by the AlgU (AlgT) stress pathway independent of flagellar regulation, whereas HAA production or export is regulated by GacS, OsmE, and AlgC (79). The genes *syfAB*, *syfR,* and *rhlA*, were induced in a microarray analysis of the transcriptome of leaf-associated B728a cells, suggesting that syringafactin, HAA, and swarming are important adaptations in *P. syringae* plant-associated fitness (8).

Quorum sensing in relation to motility

Swarming is a group effort and the expression of swarming-associated genes may be an energy drain at low cell densities. To coordinate gene expression with cell density, bacteria utilize quorum sensing through low molecular weight molecules called autoinducers (100).

These autoinducers are passively or actively secreted, and exhibit positive feedback that increases their production (100). Once a minimum threshold of autoinducers is reached, the autoinducers initiate signal transduction pathways that result in a group-coordinated change in gene expression (100). Acyl-homoserine lactones (AHLs) are a major group of autoinducers utilized by gram-negative bacteria (100). Quorum sensing is involved in swarming regulation and its involvement in biosurfactant production in *P. aeruginosa* is well understood.

Loss of quorum sensing results in the inability to swarm among several Pseudomonads (85). In *P. aeruginosa* quorum sensing regulates rhamnolipid production, with upregulation resulting in hyperswarming (94, 101). Some research suggests that additional density-dependent upregulation of *P. aeruginosa* swarming occurs due to nutrient depletion at higher densities (92). In B728a, loss of genes involved in AHL synthesis and regulation, namely *aefR*, *ahlI*, and *ahlR*, resulted in hyperswarming, demonstrating that AHL production represses swarming motility (102, 103). AHL-mediated repression of swarming motility in B728a was also associated with increased survival on leaves (104) and decreased virulence on leaves and bean pods (102).

In this dissertation we evaluate the role of light sensing in *P. syringae,* with a specific focus on how three photosensory proteins regulate light-mediated responses. We hypothesize that light is a significant environmental cue for *P. syringae* during colonization and infection of phyllosphere tissues, including leaves and fruits. In this study we characterize the role of BphP1 and LOV in regulating swarming motility in response to red and blue light, identify downstream components in the BphP1/LOV signal transduction pathway, and evaluate the contribution of BphP1 and these downstream components to colonization and virulence. Lastly, we characterize the role of light in regulating a variety of swarming-associated phenotypes and explore how

surfactants, motility structures, and global regulators form a swarming-associated regulatory network.

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CHAPTER 2. LIGHT REGULATES SWARMING MOTILITY, BUT NOT LIGHT SENSITIVITY, THROUGH A BACTERIOPHYTOCHROME AND LOV PROTEIN IN *PSEUDOMONAS SYRINGAE* PV. SYRINGAE B728A

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These included the phylogenetic analysis of the bacteriophytochromes, the impact of light on swarming motility of the single-gene deletion mutants, and the elucidation of the integrated signal-transduction pathway).

Abstract

Pseudomonas syringae is unusual among heterotrophic bacteria because it encodes three photoreceptors, suggesting that light sensing is an important adaption for its lifecycle. It encodes two red and/or far-red light-sensing bacteriophytochromes and a blue light-sensing LOV protein. In this study we demonstrate that the bacteriophytochrome BphP1 and LOV function together to regulate swarming motility in response to red+far-red and blue light. BphP1 negatively regulates swarming motility in response to blue and red+far-red light through two independent pathways, as demonstrated by the hyperswarming in red+far-red light when the *bphOP1* operon is deleted and reduced swarming in blue light when *bphOP1* is overexpressed. BphP1 blue-light-mediated repression is attenuated by LOV, as shown by the finding that BphP1 blue-light sensing occurred only when *lov* was deleted. The Δ*bphP1* and Δ*lov* mutants were not altered in flagellar swimming, biosurfactant production, or rate of swarming motility, indicating that BphP1 and

LOV did not regulate these components of swarming. We also demonstrate that *P. syringae* is sensitive to light based on observations that cell density was reduced in stationary phase during growth in continuous light as compared to continuous dark. Moreover, osmotic stress amplified this light sensitivity, indicating that osmotic stress may increase light toxicity. BphP1 and LOV, as well as the second bacteriophytochrome, BphP2, were not involved in light sensitivity as their loss did not influence cell density in the light. Although BphP2 is unique among bacteriophytochromes in having tandem histidine kinase (HisKA-HWE) domains, we were unable to identify a physiological function for BphP2. This work provides the first evidence for blue light sensing by a bacteriophytochrome in any organism and for a physiological function of a bacteriophytochrome in *P. syringae*.

Introduction

Light is an important environmental signal providing organisms with diurnal, seasonal, and positional cues. Changes in quality and quantity of light are detected by both eukaryotes and prokaryotes through photoreceptors, which are grouped into six families based on their interacting chromophores (2). For all six families, a chromophore binds the photoreceptor and initiates a change in conformation following excitation by a specific wavelength of light (2). The six families include red and/or far-red light-absorbing phytochromes (3) and five types of blue light-absorbing proteins: rhodopsins (4, 5), which also respond to green light, cryptochromes (6), light oxygen and voltage sensors (LOV) (2, 7-9), blue-light sensing FAD (BLUF) proteins (10), and photoactive yellow proteins (PYP) (11). These photoreceptors often encode C-terminal output domains such as histidine kinase, DNA-binding and GGDEF/EAL domains (12). These proteins have been well studied in plants and cyanobacteria and over the last two decades have been discovered with increasing frequency in heterotrophic bacteria through genome-wide

screens (13). The discovery of photoreceptors in non-photosynthetic bacteria was initially surprising because these bacteria do not need to utilize light as an energy source; only in recent years have studies begun to provide insights to the physiological functions of photoreceptors in bacteria.

The foliar plant pathogen *Pseudomonas syringae* is found in a range of habitats where light may have a significant role in survival, including in crops, snowpack, waterways, and soil (14). *P. syringae* is unusual among heterotrophic bacteria in encoding three photoreceptors, suggesting that light sensing is an important adaptation in *P. syringae*'s lifecycle. Two of the encoded photoreceptors are bacteriophytochromes (15); heterotrophic bacteria that encode two bacteriophytochromes are fairly unusual. The third photoreceptor is a LOV protein (16). The changes in light that *P. syringae* encounters on leaf surfaces likely correlate with environmental changes such as fluctuations in water availability and temperature, which are known to influence its survival and behavior (17-20). *P. syringae* also encounters plant defenses that are light regulated, including the sensitivity of plants to pathogen-associated molecular patterns (PAMPs); this sensitivity fluctuates depending on the time of day (21, 22). We hypothesize that the photoreceptors in *P. syringae* enable it to better adapt to its environment by sensing cues dictated by the quality and quantity of light.

Bacteriophytochromes are a class of red and far-red light sensing phytochromes encoded by bacteria (23). They are the most abundant photoreceptors found in bacteria and are present in 17% of all sequenced bacterial genomes (23). They convert between two stable conformations, a red light-absorbing Pr form and a far-red light-absorbing Pfr form; the conversion is induced when the associated light-absorbing chromophore, biliverdin (BV), is excited (13, 15). BV associates with the N-terminal bilin-binding domain of bacteriophytochromes through a Schiff-

base linkage with a conserved histidine in the GAF domain (13). This association with BV through a histidine is much like the structure of the closely related fungal phytochromes (24); in contrast, cyanobacterial phytochromes (Cphs) associate with the chromophore phycocyanobilin through a conserved cysteine in the GAF domain and are more closely related to plant phytochromes (24). Beyond the role of bacteriophytochromes in photosynthesis (25-27), phytochrome proteins encoded by cyanobacteria regulate phototaxis (28, 29), cAMP levels (30), and photolyase activity (31); in contrast, little is known of physiological function of bacteriophytochromes in heterotrophic bacteria.

The two bacteriophytochromes encoded by *P. syringae* are designated BphP1 and BphP2 and are composed of a N-terminal bilin-binding domain and a C-terminal histidine kinase domain, the latter of which is autophosphorylated when active (15). The gene *bphP1* is transcribed with *bphO,* which encodes the heme oxygenase BphO; this enzyme converts heme into BV (15). The biochemical properties of BphP1 in *P. syringae* pv. tomato DC3000 have been well studied and indicate that BphP1 is active in the Pfr form and requires BV for autophosphorylation of the conserved histidine, amino acid 532, when exposed to red light (690 nm) (15). Beyond its activity as a heme oxygenase, BphO is involved in folding BphP1 and incorporating BV into BphP1 (32). The gene *bphP2* is transcribed with *bphR,* which encodes the putative response regulator BphR; BphP2 is resistant to purification and this has limited the study of its biochemical properties (32).

Beyond the biochemical properties and the role of bacteriophytochromes in cyanobacterial photosynthesis, little has been elucidated of the function of phytochromes encoded by prokaryotes. The cyanobacterial phytochromes Cph1 and Cph2, which show a noteworthy similarity to plant phytochromes, promote growth under far-red light and red light,

respectively, in *Synechocystis* sp. strain PCC 6803 (33). Surprisingly, in addition to its red light activity, Cph2 inhibits phototaxis towards blue light (34). Cph2 has an unusual structure with three GAF domains instead of a histidine kinase domain; two of the GAF domains have bilin-binding sites allowing for the possibility of interactions with multiple types of chromophores (35). The blue light-sensing capability of Cph2 is further supported by the greater absorption in blue than red light (36). The function of bacteriophytochromes has been demonstrated in only in two organisms, *Deinococcus radiodurans* and *Pseudomonas aeruginosa*. In the extremophile *D. radiodurans* a bacteriophytochrome initiates production of the pigment carotenoid deinoxanthin in response to red and white light through signal transduction with the response regulator BphR (13). Production of deinoxanthin most likely protects *D. radiodurans* from light-induced damage (13). The animal pathogen *P. aeruginosa* induces expression of its bacteriophytochrome in a growth phase-dependent manner through RpoS; transcriptome analysis also indicates that it is regulated by LasR, suggesting a role in the RpoS/Las quorum-sensing network (37, 38). Additionally, the heme oxygenase required for function of the bacteriophytochrome independently influences heat tolerance and pyocyanin production (38).

LOV proteins are common to bacterial communities and are present in 10-15% of sequenced bacterial metagenomes representing a variety of habitats including marine, soil, sediment, air, hot spring, and mammalian gut (39). LOV proteins respond to blue light through the chromophore flavin mono nucleotide (FMN), which forms a covalent bond with a cysteine residue, forming a cysteinyl-flavin adduct when excited by blue light (16, 40). The formation of this covalent bond initiates a conformational change in the histidine kinase output domain, which requires a second reactive cysteine for autophosphorylation of the conserved histidine when exposed to blue light (40). The LOV protein encoded by *P. syringae* also has a C-terminal

response regulator (REC) domain; this LOV-HK-REC domain structure is found almost exclusively in plant pathogens, including the *Xanthomonas axonopodis* pv. citri LOV for which the physiological function has been studied (41). The only evidence thus far for phosphate transfer from the HK to the REC domain of LOV was generated with *P. syringae* pv. tomato DC3000 LOV when the two domains were expressed separately (42).

LOV proteins are not found in cyanobacteria and have only recently been studied in heterotrophic bacteria, so little is known beyond their biochemical properties. *P. syringae* pv. tomato DC3000 LOV regulates resistance to oxidative stress and motility through regulation of *fliC* (43) and represses growth on leaves, possibly via effects on the expression of multiple sigma factors (44). In the plant pathogen *Xanthomonas axonopodis* pv. citri, LOV regulates motility, attachment to leaves, extracellular polysaccharide (EPS) production, and biofilm formation (41), and influences disease symptom development and virulence (41). The *Rhizobium leguminosarum* LOV protein also regulates EPS production and nodule formation (45). The *Brucella abortus* LOV protein regulates cell proliferation in macrophages in response to blue light, which may be a method to prepare for infection of new hosts (42). The *Caulobacter crescentus* LovK/LovR two-component system regulates cell-to-cell attachment and attachment to abiotic surfaces (46) and functions in the general stress pathway through coordination with the stress-sensing two-component system PhyK-PhyR (47). An interaction with PhyR-like proteins may be conserved across species, as LOV in the marine bacterium *Erythrobacter litoralis* HTCC2594 interacts with two PhyR-like response regulators through signal transduction (48). These results seem to be consistent with a model suggesting that blue light sensing proteins regulate the choice between a single cell motile state and a multicellular sessile state (23) based on the LOV regulation of

motility in some organisms and a multicellular state as reflected in the biofilm associated behaviors of attachment and EPS production in other organisms.

In this study we sought to determine the physiological traits that are regulated by light and the three photoreceptors BphP1, BphP2, and LOV in the bacterial plant pathogen *P. syringae*. We discovered that LOV and BphP1 integrate their regulation of swarming motility through both red and blue light sensing. We found that BphP1 represses swarming motility in response to red light through its histidine kinase activity, and surprisingly, also responds to blue light based on repression of swarming motility that is independent of red light. The two photoreceptor pathways are integrated based on the ability of LOV to attenuate BphP1 blue-light mediated repression of swarming. We discovered that *P. syringae* exhibits phototoxicity as reflected in a light-mediated decrease in cell density in stationary phase, but neither BphP1, BphP2 nor LOV were involved in sensitivity. Taken together, this work provides the first evidence of blue light sensing by a bacteriophytochrome, integration of LOV and phytochrome regulation in bacteria, and detectable bacteriophytochrome regulation in *P. syringae*.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains and plasmids for this study are described in Table 1. *P. syringae* pv. syringae strain B728a (49) was grown in King's B medium (50) at 25°C unless otherwise described. B728a growth was analyzed in the media King's B, Luria (51), MinA (52) amended with 10% succinate, and ½-21C (53, 54). *Escherichia coli* strains were grown in Luria medium at 37°C. Antibiotics were added at the following concentrations as needed (µg/ml): rifampin

(Rif), 50; kanamycin (Km), 50; tetracycline (Tet), 20; spectinomycin (Spc), 20; chloramphenicol (Cm), 30; and ampicillin (Amp), 50.

Table 1. Strains and Plasmids used in this study

Strain or plasmid	Description of relevant genotype	Reference or source
***P. syringae* strain**		
B728a	Wild type; Rif^r	(49)
Δ*bphOP1*	B728a Δ*Psyr_3505-3504*; Rif^r	This study
Δ*bphP1*	B728a Δ*Psyr_3504*; Rif^r	This study
Δ*bphP2R*	B728a Δ*Psyr_2385-2384*; Rif^r	This study
Δ*bphP2*	B728a Δ*Psyr_2385*; Rif^r	This study
Δ*bphP1*Δ*bphP2*	B728a Δ*Psyr_3504*Δ*Psyr_2385*; Rif^r	This study
Δ*lov*	B728a Δ*Psyr_2700*; Rif^r	This study
Δ*bphP1*Δ*bphP2*Δ*lov*	B728a Δ*Psyr_3504*Δ*Psyr_2385*Δ*Psyr_2700*; Rif^r	This study
Δ*lov*Δ*bphP1*	B728a Δ*Psyr_2700*Δ*Psyr_3504*; Rif^r	This study
***Escherichia coli* strain**		
NEB10β	Enables high-efficiency cloning	New England Biolabs Inc., MA
Plasmids		
pTOK2T	pTOK2 with restored *lacZ* activity; Tet^r	(55)
pKD13	Template for *kan* cassette flanked by FLP recombination sites; Amp^r Km^r	(56)
pFLP2Ω	pFlp2 (57), which encodes a FLP recombinase, with a Spc^r Ω cassette (58); Amp^r Spc^r	This study
pRK2013	RP4 remobilization; Km^r	(59)
pME6041	Broad-host range vector; Km^r	(60)
pN	pME6041 with *nptII* promoter; Km^r	(55)
pN*bphOP1*	pN with *Psyr_3405-3505* under the control of its native promoter in tandem with the *nptII* promoter; Km^r	This study
pN*bphOP1*H530L	pN*bphOP1* with a point mutation in histidine 530	This study
pBphP1	pME6041 with *Psyr_3504* under control of its native promoter; Km^r	This study
pN*bphP2R*	pN with *Psyr_2385-2384* under control of its native promoter in tandem with the *nptII* promoter; Km^r	This work
pLOV	pME6041 with *Psyr_2700* under control of its native promoter; Km^r	This study

Phylogenetic analysis

A phylogenetic analysis of prokaryotic phytochromes was performed by using ClustalW (61) to align the complete sequences of PHY-domain containing proteins that were selected to represent diverse taxa, including photosynthetic bacteria. *P. syringae* BphP2 encodes two histidine kinase domains making it as much as 250 amino acids larger than the other phytochromes; the non-aligning end regions were removed to better compare protein relatedness. Using Akaike Information Criterion (AIC) and MrBayes, a mixed amino acid model with gamma-distributed rates and without a molecular clock was determined to be the best-fit model (62). Sampling was performed using MrBayes over 1,000,000 generations with a burn-in of 2,500. MrBayes starts by randomly generating trees and as the program progresses it narrows in on the most supported arrangement based on the selected model; setting a burn-in of 2,500 results in the first 2,500 trees being discarded to prevent the least-supported trees from skewing the posterior probabilities. TreeAnnotator of Beast Software was then used to visualize the supported tree (63).

To compare the LOV domains encoded by heterotrophic bacteria, ClustalW (61) was used to align the sequences of the 200-amino acid LOV domains of 20 non-photosynthetic bacteria that were selected to represent diverse taxa. Using the method described above, the Whelan and Goldman (WAG) substitution model with no clock was determined to be the best-fit model. The same sampling as the phytochromes was used with MrBayes, and TreeAnnotator of Beast Software was used to construct the supported tree (63).

Construction of mutants and strains expressing *bphP1, bphP2,* and *lov* on plasmids

Single-gene and operon deletion mutants were constructed as described previously (55). In short, regions upstream and downstream of the target site were amplified using PCR and were ligated to a PCR-amplified kanamycin (*kan*) cassette of pKD13 (56), which was flanked by FRT sites, using splice-overlap-extension (SOE) PCR (64). This generated a single fragment with the *kan* cassette between the upstream and downstream components. This fragment was cloned into pTOK2T (55) using the SmaI site. The resulting construct was introduced into B728a by conjugation with the helper plasmid pRK2013 (59). Km-resistant colonies were then screened for double recombinants. After confirmation of the mutants by PCR, the *kan* cassette was removed by introduction of pFlp2Ω (1), which encodes the Flp recombinase, to target the FRT sites of the *kan* cassette. pFlp2Ω was removed by counter-selection with 10% sucrose. Deletion of the target gene was confirmed by PCR and sequencing. The Δ*bphP1*, Δ*bphP2*, and Δ*lov* mutants, hereafter referred to simply by the name of their mutations (Table 1), were then used to construct the mutants Δ*bphP1*Δ*bphP2*, Δ*bphP1*Δ*bphP2*Δ*lov*, and Δ*bphP1*Δ*lov*. For example the pTOK2T construct containing the SOE PCR fragment for deletion of *bphP1* was introduced into Δ*bphP2* by conjugation and then selected for and confirmed as described above. Primers utilized for the construction of each mutant can be found in Table 2.

To construct expression plasmids to evaluate complementation of the Δ*bphOP1* and Δ*bphP2R* mutants, *bphOP1* and *bphP2R* were amplified with their native promoters by PCR and cloned into the EcoRV site of pN (55). pN is pME6041 (60) with a 733-bp fragment containing the *nptII* promoter inserted upstream of the MCS. This resulted in a fusion of *nptII* with the respective native promoters. These constructs were designated pN*bphOP1* and pN*bphP2R* (Table 1). Site-directed mutagenesis of pN*bphOP1* was performed using a QuikChange II XL

site-directed mutagenesis kit (Aligent Technologies, Santa Clara, CA). The resulting construct was designated pN*bphOP1*H530L. The plasmids pLOV and pBphP1 were constructed as described in Wu et al. (1). Primers utilized for construction of the complementation plasmids can be found in Table 2.

Table 2. Primers used for this study

Primer	Sequences
Primers for constructing deletion mutants	
bphOP1-flanking region 1-F	5'-GACAACGACGGTTTTCAGGTCAGC-3'
bphOP1-flanking region 1-R	5'-AGCCTACACAATCGCTCAAGACGTGCTCTTGAGTGTGCAATGGGCAGA-3'
bphOP1-flanking region 2-F	5'-AATATCCGGGTAGGCGCAATCACTCGCTTGCTCAGGCTTGCAGTG-3'
bphOP1-flanking region 2-R	5'-GTGGCTACGCAAGTGCTGTTGG-3'
bphP1-flanking region 1-F	5'-GAAGCTAACCAACACGCCTTCTG-3'
bphP1-flanking region 1-R	5'-AGCCTACACAATCGCTCAAGACGTGAGTTCCGCTTCACGGTGCCAATG-3'
bphP1-flanking region 2-F	5'-AATATCCGGGTAGGCGCAATCACTCACAATTGGCCAGAAGGACTTC-3'
bphP1-flanking region 2-R	5'-TTCAGCGTTGTCACGCAAGC-3'
bphP2R-flanking region 1-F	5'-GCCCATTCGCGACCTTCTCG-3'
bphP2R-flanking region 1-R	5'-AGCCTACACAATCGCTCAAGACGTGCCAGCCGCTGCTTGGTACG-3'
bphP2R-flanking region 2-F	5'-AATATCCGGGTAGGCGCAATCACTTCCGCTGGCCGTATTGTCAAAG-3'
bphP2R-flanking region 2-R	5'-CGCCAACCTGATGAACCCGATG-3'
bphP2-flanking region 1-F	5'-ACGCTATGCTTCGCGCTTCAAC-3'
bphP2-flanking region 1-R	5'-AGCCTACACAATCGCTCAAGACGTCAATGCTGATTCCCTGGCCAGAC-3'
bphP2-flanking region 2-F	5'-AATATCCGGGTAGGCGCAATCACTGCATCCAGCATGTCCTTGATC-3'
bphP2-flanking region 2-R	5'-CTTCAAGGACAAGGTAGCGAC-3'
lov-flanking region 1-F	5'-TCCGGGCCAGTATTGCGGCAATAG-3'
lov-flanking region 1-R	5'-AGCCTACACAATCGCTCAAGACGTCATCCGTGTCGTTTCTACCGCCGC-3'
lov-flanking region 2-F	5'-AATATCCGGGTAGGCGCAATCACTCGGCAATATGTATGACTTGCTGTTCAC-3'
lov-flanking region 2-R	5'-CAGAATCGCACTCCAGGAGATTCG-3'
kan cassette-F	5'-ATTGTGTAGGCTGGAGCTGCTTC-3'
kan cassette-R	5'-CCATGGTCCATATGAATATCCTCC-3'
Primers for constructs expressing *bphOP1*, *bphP1*, *bphP2R*, and *lov* in the deletion mutants	
bphOP1-F	5'-GCGGTGGCAAACCGTCGTCTTA-3'
bphOP1-R	5'-GCGCGAGGTACTTCCAGCGAAC-3'
bphP1-F	5'-CCGGAATTCATGTTTCGAGCACTGGCTCGA-3'
bphP1-R	5'-GCCGGATCCTCAAACCGCCATTGGCACCGT-3'
Promoter *bphO*-F	5'-GAAGATCTCCACGCGAGCTGGCCTTTTCAGCGTTG-3'
Promoter *bphO*-R	5'-CATGCCATGGCCAGGAAGGCTCTTGAGTGTG-3'

Table 2 continued

bphP2R-F	5'-GAGCGCCTCATCCAGGTCTCCA-3'
bphP2R-R	5'-GCCGCCGATCCGTCAGTAATCA-3'
Promoter *lov*-F	5'-CCGGAATTCGTAAGCGTTTCGGGAATGCGT-3'
Promoter *lov*-R	5'-CATGCCATGGTCAGGCGATACCGTTCGGCCCGTC-3'
Primers for site-directed mutagenesis	
*bphOP1*H530L-F	5'-GTGTCCCTTGACTTGCGTAACCCG-3'
*bphOP1*H530L-R	5'-CGGGTTACGCAAGTCAAGGGACAC-3'
Primers for construction of His_6-tagged BphP1	
*bphP1*BamHI-F	5'- GCCGGATCCATGAGCCAACTCGACAAAGACGCC-3'
*bphP1*NotII-R	5'- ATTTGCGGCCGCTCAAACCGCCATTGGCACCGTGAA-3'

*underlined portions of the primer sequence indicate the region that binds to the *kan* cassette

Growth assay

Three independent 5 ml cultures of each strain were grown in King's B broth in 25 ml glass test tubes with shaking at 25^0C to late-log phase. The cells were then harvested, washed twice with sterile, nanopure water and normalized to an optical density at 600 nm of 1.5 using a spectrophotometer (Nanodrop2000c, ThermoFisher Scientific Inc., Waltham, MA). Ten µl containing approximately $4x10^6$ cells were added to 90 µl of broth in a 96-well microtiter plate. Two replicate microtiter plates were prepared to compare light and dark conditions. Each plate was wrapped in parafilm. The dark treatment was enclosed in aluminum foil while the light treatment was exposed to white light using fluorescent bulbs (Ecolux F40C50-Eco, General Electric Co., Fairfield, CT) at a distance such that the light intensity was 30 µmol $m^{-2}s^{-1}$. For blue light treatments bilirubin bulbs (Interlectric Corp, Warren, Pennsylvania) were used and the cells were exposed at a distance such that the light intensity was 21 µmol $m^{-2}s^{-1}$. The cells were grown at 25^0C with shaking at 250 rpm and monitored using an EL 340 Microplate Biokinetics Reader (Bio-tek Instruments, Winooski, VT) by normalizing the optical density at 405 nm and 630 nm to reduce the impact of condensation on the lid of the microtiter plate. Growth studies were repeated a minimum of three times.

Motility

Swimming motility was evaluated using circular petri plates (100 x 15 mm) containing King's B medium with 0.25% agar, whereas twitching motility was evaluated using King's B medium amended with 0.05% tetrazolium chloride and 1% agar. The plates were allowed to dry on the bench top overnight. Wild-type cultures were grown and the cells harvested as described for evaluating growth. For swimming motility, stab inoculation was used with a pipette tip containing a 2 µl suspension containing $8x10^5$ cells. The plates were both enclosed in parafilm and one placed under white light and the other enclosed in aluminum foil. The plates were incubated at 22-23^{0}C for approximately 24 h after which they were photographed.

Analysis of swarming motility was performed using square petri plates (100 x 100 x 15 mm) containing 30 ml or circular petri plates (100 x 15 mm) containing 25 ml of King's B medium with 0.4% agar. The moisture of the plates was critical for obtaining reproducible swarming, so plates were prepared according to a routine protocol. Petri plates containing media were allowed to dry with the lids off in a laminar flow hood for 35-45 minutes with the bottom 8 in of the rear panel blower covered; alternatively, they were dried on a bench top overnight. Cultures were grown and the cells harvested and prepared as described for the growth assay. The swarming motility of a single strain was examined as described for the swimming motility assay, except that plates were subjected to three distinct light treatments, white light, red+far-red light, and blue light, and were incubated at 22-23^{0}C for 24-36 h. Swarming motility exhibited high plate-to-plate variability, therefore multiple strains were compared by placing strains on solid media in a square petri plate and arranging them such that each strain was represented at least once in every row and every column. By placing five drops of each culture on a single plate, up to 5 strains could be compared in a single swarming motility test. The plates were incubated at

22-23^{0}C until the colonies showing the most expansive swarming were no less than 5 mm from the nearest colony; this prevented overlap and interference between colonies and typically occurred after 10-14 h. For blue (470 nm), red (680 nm), and far-red (750 nm) light, light-emitting diodes from Marubeni America Corporation were used with light intensities of 5, 10, and 0.8 µmol $m^{-2}s^{-1}$ respectively. After incubation the plates were photographed and the lateral surface area of each colony was quantified using the area selection tools of Adobe Photoshop and the pixels corresponding to the total surface area of a colony were recorded (1). Statistical analyses included Student's *t* tests for two-strain comparisons and analysis of variance (ANOVA) for multiple-strain comparisons. Each comparison was repeated a minimum of three times.

To quantify the number of cells present in each swarm colony, swarm plates were prepared as described above. After approximately 12 h the plates were photographed for surface area measurements and each colony was excised from the plate and resuspended in nanopure water for enumeration on King's B medium containing Rif.

To quantify rate of motility, 2 µl of prepared cells were inoculated onto the end of a 1.5 x 4.5-cm filter strip that had been placed on King's B medium (1.5% agar) for 5 min. The plates were incubated for 5 h at 22-23^{0}C in either white light or enclosed in aluminum foil. The filter was then removed by gentle lifting and the plate incubated for 36 h. The distance of swarming on the filter was estimated based on the location of bacterial growth on the agar medium, as in Burch et al (65). Rate per hour was determined by measuring the distance from the point of inoculation to the end of the area of bacterial growth and dividing it by the time of incubation. For each strain 4 replicates were analyzed. Quantification of motility rates was performed three times.

Expression of *bphOP1* in wild type and Δ*bphOP1*(pN*bphOP1*)

Three independent cultures of the wild type, Δ*bphOP1*, and Δ*bphOP1*(pN*bphOP1*) were grown in King's B medium overnight. Using an RNeasy Mini Kit, RNA was extracted from each culture and DNA was removed using on-column DNase I digestion (Qiagen, Venlo, Limburg). Expression of *bphP1* was measured using quantitative reverse transcription-PCR (qRT-PCR) with the Qscript one-step qRT-PCR kit (Quanta Biosciences, Gaithersburg, MD). From the cycle threshold (CT) values, relative expression was calculated using expression of the *hemD* gene as the internal control. The primers 5'-TTTCGACGTTGCGCAGTGTTTCAC-3' and 5'-AATCAGCGACACACTCATGGACGA-3' were used to evaluate expression of *bphP1*, and 5'-TGCACAGCGTTCGATCAT-3' and 5'-GCCATACTTCGTCGATCAGTT-3' were used to evaluate expression of *hemD*.

Results

P. syringae BphP proteins may be functionally redundant and LOV proteins encoded by plant-associated bacteria are closely related

A phylogenetic analysis was performed to compare *P. syringae* B728a-encoded bacteriophytochromes to those encoded by cyanobacteria and other heterotrophic bacteria (Fig. 1). Since BphP1 and BphP2 are orthologs, their evolutionary relationship can be evaluated in the same tree. A mixed amino acid model with gamma–distributed rates and without a molecular clock was determined to be the best fit model. This meant that we assumed that the rate of variation of a particular site was gamma-distributed with no enforced molecular clock. The placement of each node had posterior probabilities all above 0.7, demonstrating that the tree is highly supported. Three species utilized for this comparison encode two bacteriophytochromes:

P. syringae, *Agrobacterium tumefaciens*, and *Pseudomonas putida*. The BphP1 proteins of *A. tumefaciens* and *P. putida* cluster together, and the same is true for their BphP2 proteins; however, *P. syringae* BphP1 and BphP2 cluster together, suggesting that they are less

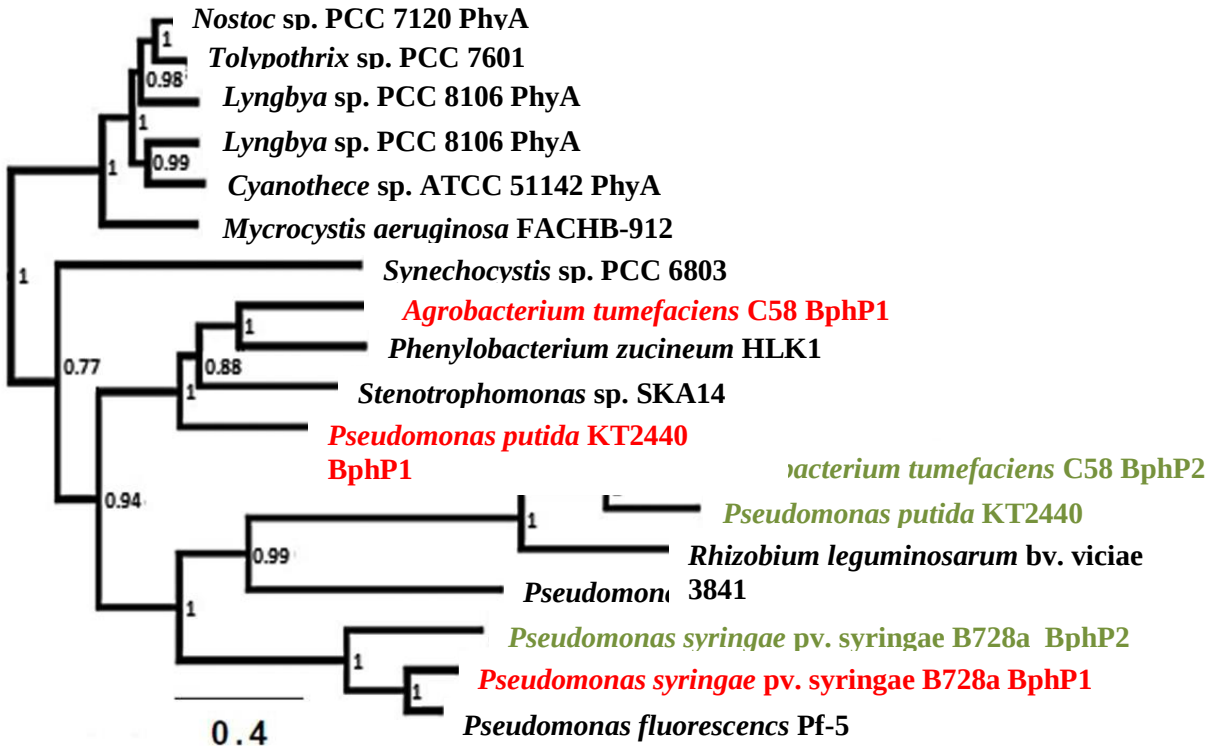

Figure 1. Phylogenetic analysis of prokaryotic phytochromes demonstrated that BphP1 and BphP2 in *Pseudomonas syringae* B728a are closely related. For each protein sequence the genus, species, strain, and protein name are included when available. The values at each node represent posterior probabilities. *Agrobacterium tumefaciens*, *Pseudomonas putida*, and *P. syringae* each encode two bacteriophytochromes which are designated BphP1 (shown in red) and BphP2 (shown in green). The accession numbers for the phytochromes, from top to bottom, are NP_487197.1, AAL76159.1, ZP_01623500.1,YP_001803399.1, ADC32653.1, NP_442237.1, NP_285374.1, NP_354963.2, YP_002131100.1, ZP_05135439.1, NP_744505.1, NP_355125.1, NP_745504.1, YP_769292.1, NP_252806.1,YP_235462.1, YP_236574.1, and YP_262277.1.

evolutionarily diverged than those encoded by other organisms. This close relationship suggests that *P. syringae* BphP1 and BphP2 have arisen from a recent gene duplication and thus could exhibit some functional redundancy. However, *P. syringae* has a second histidine kinase domain resulting in 250 amino acids that did not align with the other bacteriophytochromes and therefore

was not included in the phylogenetic analysis. The implications of this unique tandem histidine kinase domain structure are unknown because it is not found in any other sequenced bacteriophytochrome.

A phylogenetic analysis was also performed to compare the LOV domains encoded by 20 non-photosynthetic bacteria (Fig. 2). A Whelan and Goldman (WAG) substitution model without

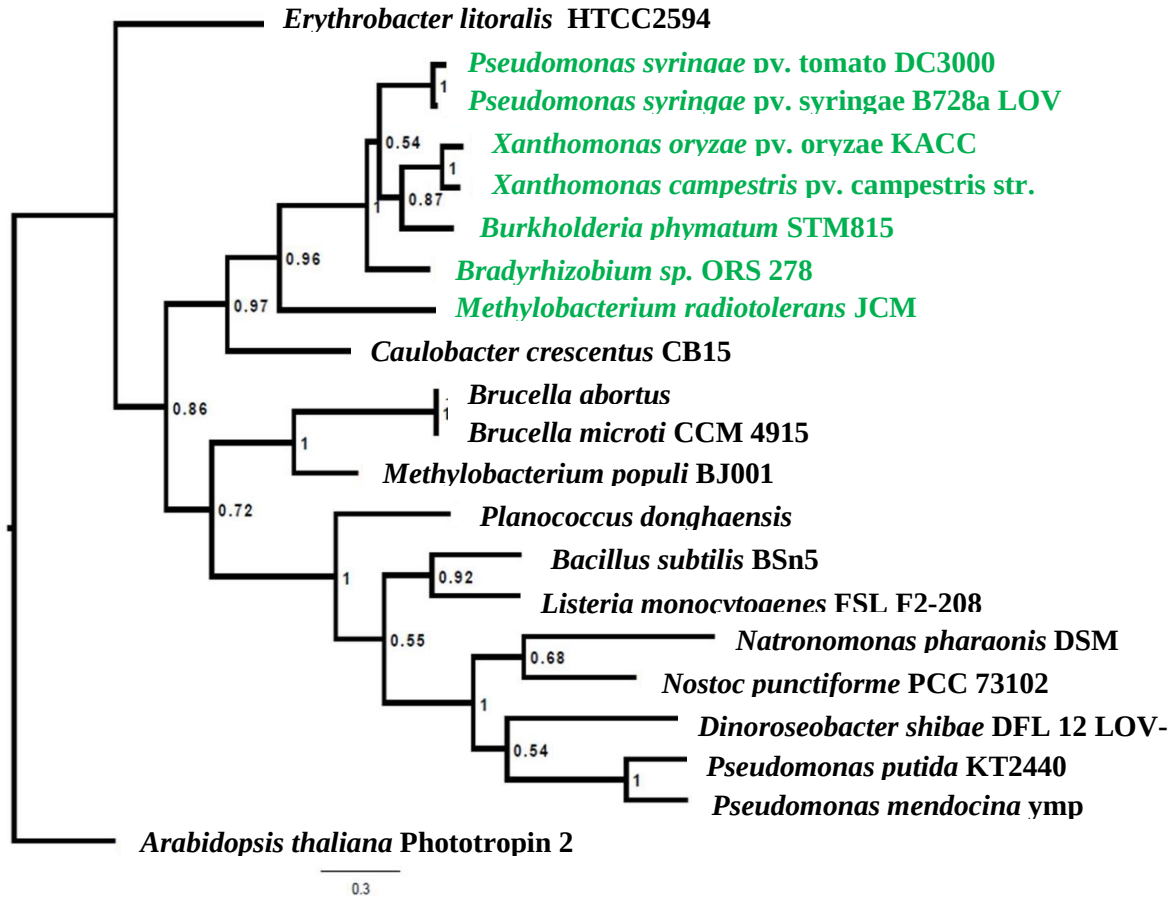

Figure 2. Phylogenetic analysis of prokaryotic LOV domains demonstrated that LOV domains from plant-associated bacteria (shown in green) are closely related. The LOV domains from 20 bacteria were analyzed and rooted using the LOV domain from *Arabidopsis thaliana* Phototropin 2. For each sequence the genus, species, strain, and protein name are included when available. The values at each node represent posterior probabilities. The accession numbers for the LOV proteins, from top to bottom, are YP_457485.1, NP_792694.1, YP_235777.1, YP_201786.1, YP_0019103150.1, YP_001858578.1, YP_001208470.1, YP_001755688.1, NP_419104.2, WP_002967295.1, YP_003105374.1, YP_001926756.1, WP_0084302040.1, YP_004204857.1, EFR85347.1, YP_327664.1, YP_001865874.1, YP_001533236.1, NP_746738.1, YP_001188769.1, NP_568874.2

a molecular clock was determined to be the best-fit model. WAG is a method that utilizes an efficient maximum-likelihood estimation approach to predict evolutionary relatedness (66). Instead of clustering with LOV domains encoded by other pseudomonads, *P. syringae* LOV clusters with plant-associated bacteria. The close relatedness of these LOV domains suggests that they are important for survival in association with the plant. Additionally, because these LOV domains cluster by the environment they are found in and not by species, they may have been shared through horizontal gene transfer. Another possibility is that the LOV domains of plant-associated bacteria functionally converged because of shared adaptations.

P. syringae B728a is sensitive to white and blue light when grown in culture, but LOV, BphP1 and BphP2 did not influence this sensitivity

Growth of *P. syringae* was analyzed in both white light and dark conditions to evaluate the effect of light on growth. To evaluate if the photoreceptors were involved in any light-regulated phenotypes, the deletion mutants Δ*bphOP1*, Δ*bphP2R,* and Δ*lov* were constructed (Fig. 3) and examined for growth. Light did not influence the rate of growth in King's B, MinA, ½-21C, or Luria media, but did reduce the cell density in stationary phase, with a particularly large impact in Luria medium (Fig 4).

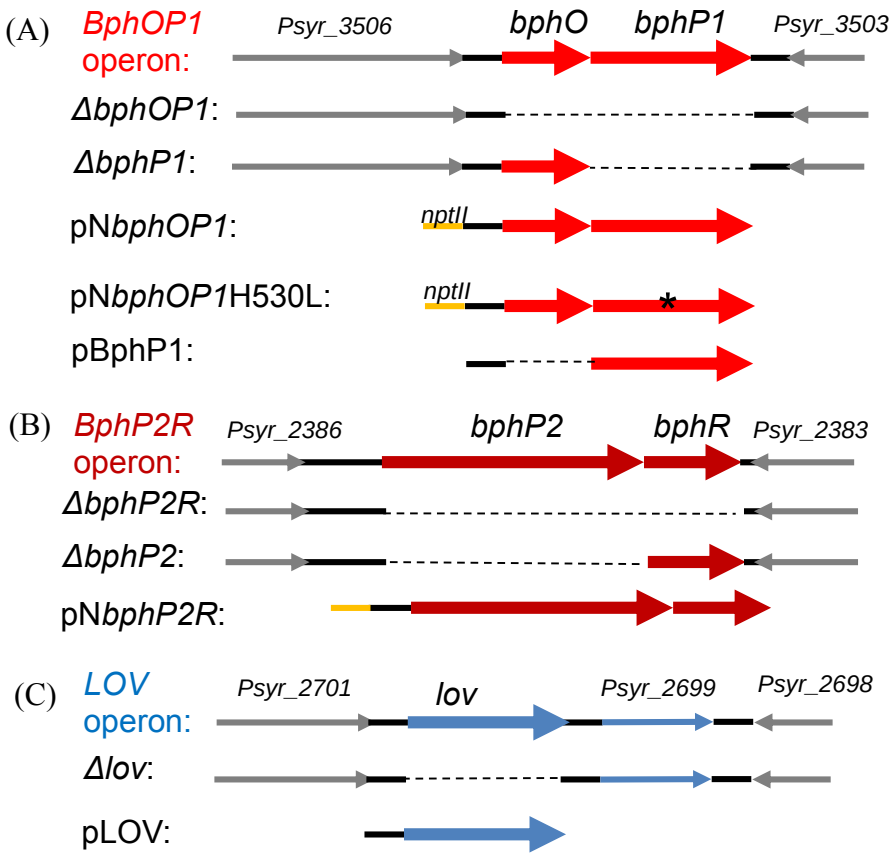

Figure 3. BphP1, BphP2, and LOV are encoded in three separate operons. (A) *bphP1* is co-transcribed with *bphO* encoding a heme oxygenase, and (B) *bphP2* is co-transcribed with *bphR* encoding a response regulator. (C) *lov* is annotated as being monocistronic, but was shown to be co-transcribed with *Psyr_2699* (see text). The deletions and potential complementation constructs are drawn below the genes.

Deletion of *lov*, *bphOP1*, or *bphP2R* did not alter growth in white light or dark conditions (Fig. 4). When growth was examined in blue light in MinA and ½-21C media, blue light also reduced cell density in stationary phase (Fig. 5), suggesting that blue light may be the component of white light that is responsible for the reduction. In blue light neither Δ*bphOP1* nor Δ*lov* were altered in growth under the tested conditions. Taken together these studies demonstrate that the LOV, BphP1 and BphP2 proteins do not influence the sensitivity of stationary phase cells to light.

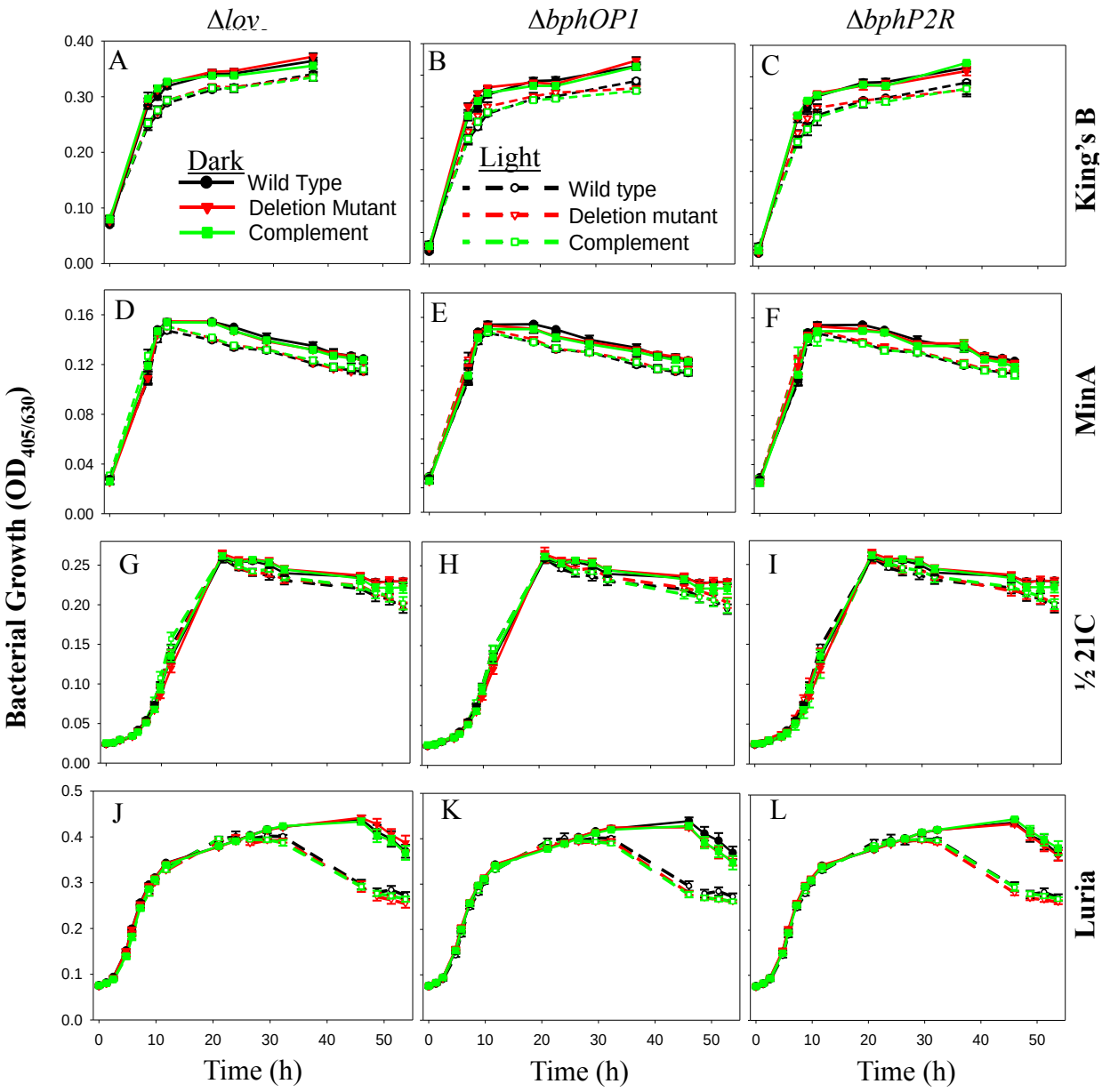

Figure 4. *P. syringae* B728a was sensitive to white light when grown in culture, but LOV, BphP1 and BphP2 did not contribute to this sensitivity. Growth of the wild type, Δ*lov,* Δ*bphOP1,* and Δ*bphP2R* along with Δ*lov*(pLOV), Δ*bphOP1*(pN*bphOP1),* Δ*bphP2R*(pN*bphP2R*) was compared in King's B (A, B, C), MinA (D, E, F), ½ 21C (G, H, I), and Luria (J, K, L) media under light and dark conditions. Values represent mean ± standard error (SE) (n = 6).

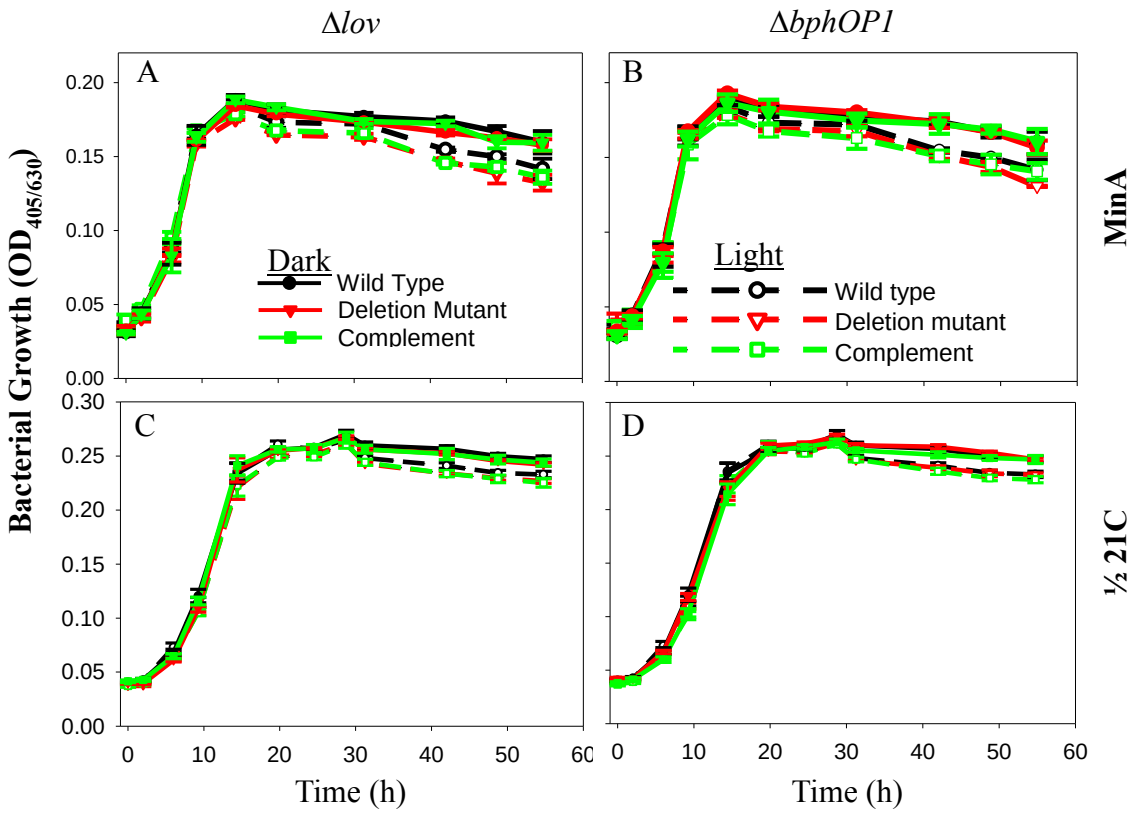

Figure 5. *P. syringae* B728a was sensitive to blue light when grown in culture, but LOV and BphP1 did not contribute to this sensitivity. Growth of wild type, Δ*lov* and Δ*bphOP1* along with Δ*lov*(pLOV) and Δ*bphOP1*(pN*bphOP1)* was compared in MinA (A, B) and ½ 21C (C, D) media under blue light and dark conditions. Values are as described in Fig. 4.

LOV is involved in responding to stress and growth under high osmolarity conditions in *C. crescentus* (46) and interacting with stress response-associated regulators in *C. crescentus* and *E. litoralis* (48). To test if LOV is similarly involved in stress responses in *P. syringae*, the growth of the wild type, Δ*lov*, and Δ*bphOP1* were evaluated in white light and dark in the presence of high osmolarity (Fig. 6). Following exposure to high osmolarity, *P. syringae* exhibits a prolonged lag phase while it accumulates compatible solutes (55); this lag phase was extended even more in the combined presence of osmotic stress and light (Fig. 6). However, Δ*lov* and Δ*bphOP1* grew like the wild type, demonstrating that they do not influence sensitivity to this combination of stresses.

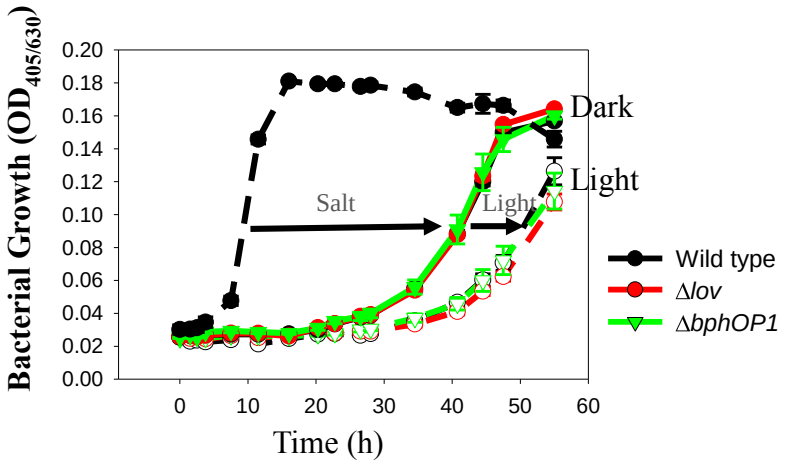

Figure 6. *P. syringae* B728a exhibited a longer adaptation period to osmotic stress in the light than in the dark, but BphP1 and LOV did not contribute to the longer adaptation time. Growth of the wild type, Δ*lov* and Δ*bphOP1* was evaluated under white light and dark conditions in MinA medium that was amended with 0.5 M NaCl. Values are as described in Fig. 4. (Dashed: dark, without NaCl; solid: dark, with NaCl; dotted: light, with NaCl).

Light represses *P. syringae* swarming motility through BphP1

In cyanobacteria, photoreceptors often regulate phototaxis as a means to optimize photosynthesis (67). Such regulation of motility led us to evaluate the impact of light on *P. syringae* swimming, swarming and twitching. By inoculating *P. syringae* cells onto plates of various agar concentrations and exposing them to either light or dark conditions, we observed that swimming and twitching motility were not influenced by exposure to light (data not shown). However, swarm plates kept in the dark showed more movement than those exposed to light (Fig. 7A). *P. syringae* forms tendrils when it swarms; therefore, Adobe Photoshop was utilized to measure the surface area covered based on pixel counts of digital images. This method demonstrated that cells in light swarmed significantly less than cells in the dark ($P<0.005$) (Fig. 7B). A similar approach to quantifying swarming was used in previous studies (68, 69).

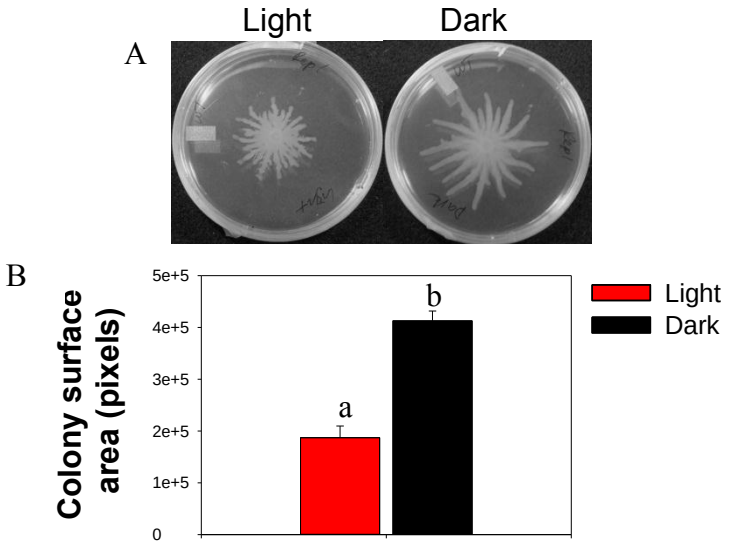

Figure 7. White light reduced *P. syringae* B728a swarming motility. The swarming of the wild type under light and dark conditions was compared (A) qualitatively and (B) quantitatively. In (B) photographs were taken and the pixel counts of the swarm colony were analyzed using Adobe Photoshop. Values are the mean ± SE (*n*=6). Letters indicate values that significantly differ from one another (P<0.01, Student's *t*-test comparing the surface area in the light versus the dark).

To evaluate the role of the phytochromes in light-mediated repression of swarming, mutants lacking the *bphOP1* and *bphP2R* operons were examined. Whereas the Δ*bphOP1* and Δ*bphP2R* mutants did not differ significantly from the wild type in their swarming in the dark, Δ*bphOP1* hyperswarmed, that is, it swarmed significantly more than the wild type in the light (Fig. 8) (P<0.05). Expressing *bphOP1* on a plasmid under the control of the *nptII* promoter, which was placed just upstream of the native *bphOP1* promoter, reduced swarming to levels lower than the wild type when introduced into Δ*bphOP1*; this tandem *nptII-bphOP1* promoter on

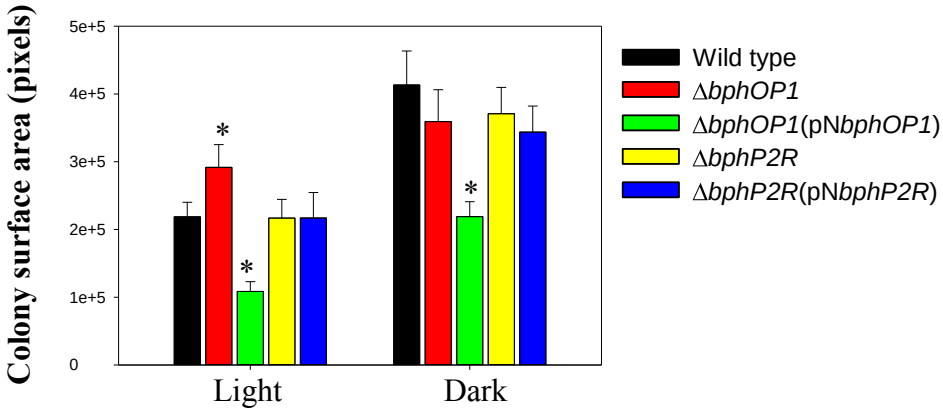

Figure 8. Swarming motility was repressed under white light by BphP1 and not affected by BphP2, whereas overexpression of *bphOP1* led to repression of swarming. *, values differ significantly from the wild type in the corresponding light/dark conditions ($P<0.05$, two-way ANOVA based on strain and light/dark conditions, $n=6$). Values represent mean ± SE.

a low-copy plasmid conferred higher *bphOP1* expression than did the *bphOP1* promoter on the chromosome (Fig. 9). This higher expression probably explains the enhanced repression of swarming by Δ*bphOP1*(pN*bphOP1*). Δ*bphP2R* and Δ*bphP2R*(pN*bphP2R*) did not differ from the wild type in the light, demonstrating that BphP2 is not involved in light-mediated repression of swarming motility, and did not differ in the dark (Fig. 8). The operon mutants presented here give results that are consistent with deletion mutants lacking only the bacteriophytochrome genes, Δ*bphP1* and Δ*bphP2* (1).

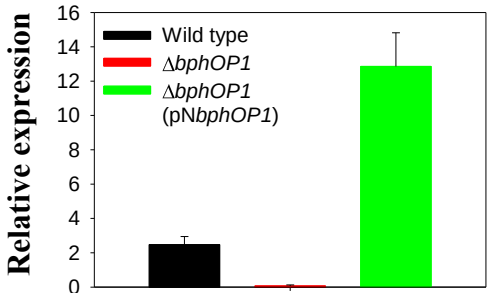

Figure 9. *bphP1* was expressed 5-fold more in Δ*bphOP1*(pN*bphOP1*) than in the wild type. qRT-PCR was utilized to compare the relative expression of *bphP1* in the wild type, Δ*bphOP1*, and Δ*bphOP1*(pN*bphOP1*). Values represent mean ± SE ($n=3$).

BphP1 and LOV regulate swarming through red and blue light, respectively, with the deletion of *bphP1* being epistatic to the deletion of *lov*

To determine the impact of distinct wavelengths on swarming motility, the swarming of the wild type and various mutants were evaluated under white, blue, red+far red, and dark conditions. Like Δ*bphP2R* (Fig. 8), Δ*bphP2* swarmed at the same level as the wild type under white light (Fig. 10). Δ*bphP2* also swarmed like the wild type under all other light conditions,

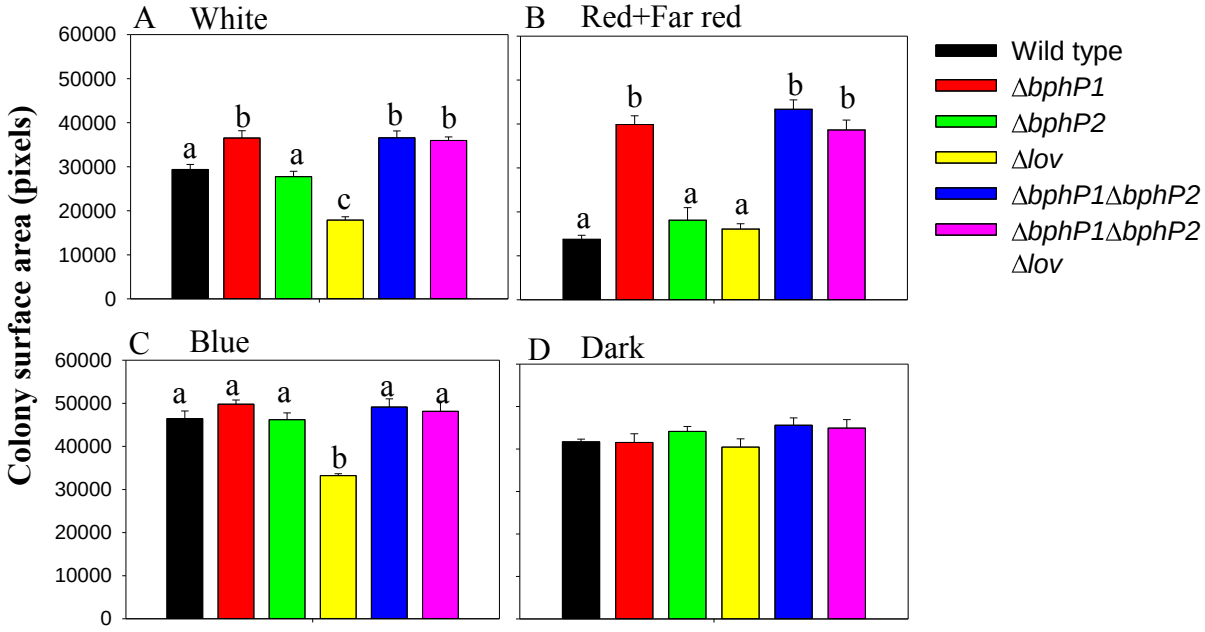

Figure 10. Swarming motility was repressed by BphP1 through red-light sensing and induced by LOV through blue-light sensing. The swarming of the wild type, Δ*bphP1,* Δ*bphP2,* Δ*lov,* Δ*bphP1*Δ*bphP2,* and Δ*bphP1*Δ*bphP2*Δ*lov* were compared in (A) white light, (B) blue light, (C) red+far-red light, or (D) dark conditions. After 10-15 h, photographs were taken and quantified as described in Fig. 7. Values represent mean ± SE and those in the same figure indicated by the same letter do not differ significantly (P<0.01, ANOVA, *n*=5). The absence of letters indicates the absence of significant differences among the strains. This figure was published in collaboration with Liang Wu (1).

providing further support that BphP2 is not involved in regulating swarming motility. When subjected to either white (Fig. 10A) or blue (Fig. 10C) light, Δ*lov* showed reduced swarming motility, demonstrating that LOV positively regulates swarming motility through blue-light

sensing. Δ*bphP1* exhibited hyperswarming not only under white light (Fig. 10A), like Δ*bphOP1* (Fig. 8), but also observed under red+far red light (Fig. 10B), demonstrating that BphP1 responds to red and/or far-red light to repress swarming motility. Quantification of the number of cells in the swarm colonies confirmed that the differences in swarming based on the colony surface area was indeed due to swarming and not growth, since the wild type, Δ*bphOP1* and Δ*lov* had similar cell numbers (Fig. 11A, B) for swarm colonies that differed in surface area (Fig. 11C, D).

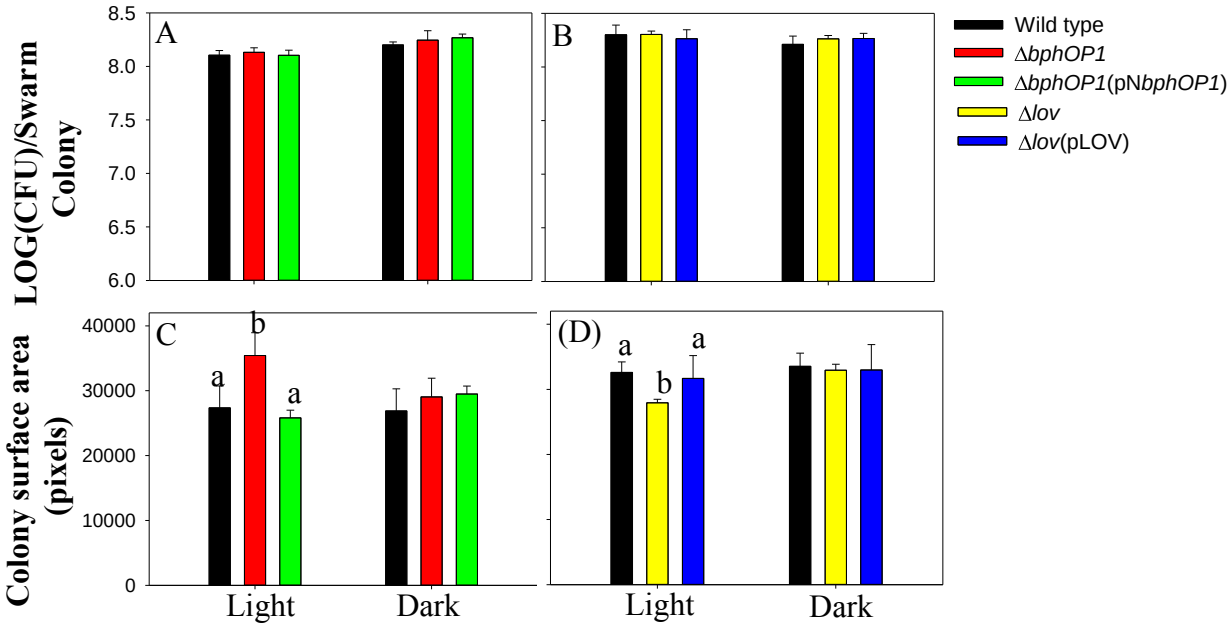

Figure 11. Differences in surface area of swarm colonies were not due to differences in growth. Swarming assays were performed as described in Fig. 10 under white light and dark conditions and the cell number per colony determined. Values in the same light condition indicated by the same letter do not differ significantly (P<0.05, ANOVA, *n*=5). The absence of a letter indicates the absence of significant differences among strains within a given condition.

To further evaluate the role of BphP1 in light sensing, *bphOP1* was expressed in Δ*bphOP1* and was evaluated under the three light conditions and the dark. Under all conditions, introduction of pN*bphOP1* caused swarming to be repressed to levels lower than the wild type (Fig. 12), presumably due to overexpression of *bphOP1* (Fig. 9). The swarming of

Δ*bphOP1*(pN*bphOP1*) presented here is consistent with the behavior of Δ*bphP1*(pBphP1) (1). These results confirm that BphP1 is involved in the light-mediated repression of swarming through red-light sensing, and suggest that overexpression of *bphOP1* further represses swarming.

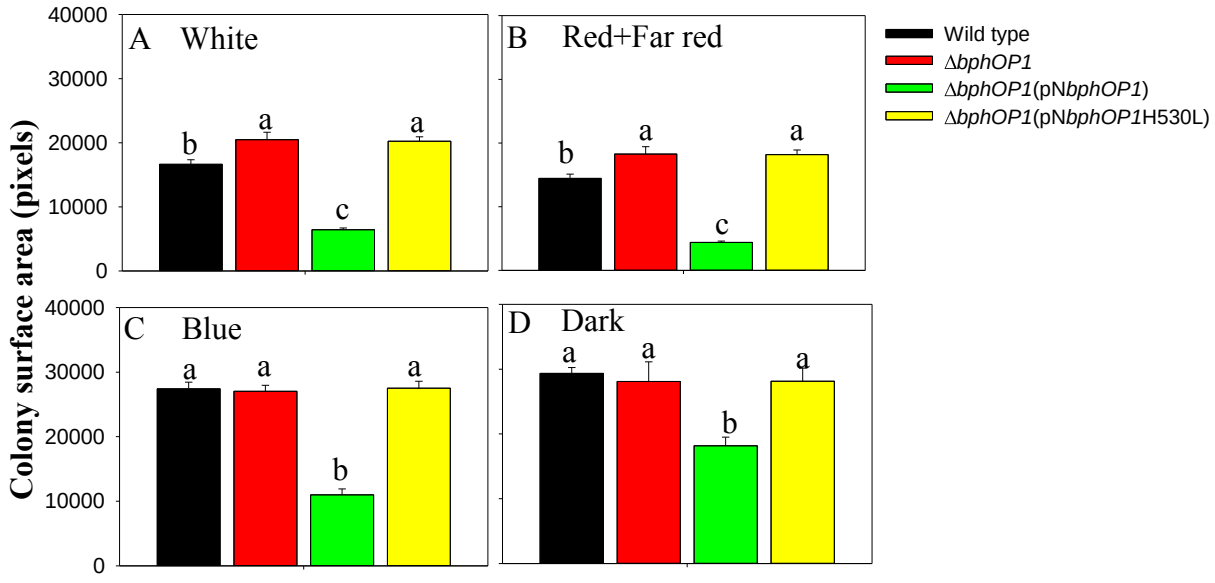

Figure 12. BphP1 repressed swarming motility through histidine kinase activity in response to red light. Swarming was evaluated under (A) white, (B) red+far red, (C) blue and (D) dark conditions. Values are as described in Fig. 10. ($P<0.05$, one-way ANOVA, $n=5$)

Our phylogenetic analysis of BphP1 and BphP2 (Fig. 1) suggested the possibility of gene duplication. However, we did not observe a detectable phenotype for Δ*bphP2* or Δ*bphP2R*. Moreover, the double mutant Δ*bphP1*Δ*bphP2* exhibited similar motility to Δ*bphP1* under all light conditions (Fig. 10). Collectively, these results indicate that BphP1 and BphP2 are not functionally redundant and that BphP2 does not function in swarming motility under these conditions.

Δ*bphP1*Δ*bphP2*Δ*lov* exhibited increased swarming motility under white light (Fig. 10A) and red+far-red light (Fig. 10B). The similarity in swarming of Δ*bphP1*Δ*bphP2*Δ*lov* to Δ*bphP1* and Δ*bphP1*Δ*bphP2* but not to Δ*lov* in white, red+far-red, and blue light demonstrates that the

loss of *bphP1* was phenotypically dominant to the loss of *lov*. Moreover, the finding that Δ*bphP1*Δ*bphP2*Δ*lov* had comparable swarming to the wild type in blue light despite the reduced swarming of Δ*lov* suggests that BphP1 is capable of sensing blue light, which is unexpected given the known role of bacteriophytochromes only in red and far-red light sensing.

BphP1 represses swarming motility through histidine kinase activity in response to red light

To determine if the histidine kinase domain of BphP1 is critical to repressing swarming motility, we introduced a point mutation in the predicted autophosphorylation site (15), amino acid 530, that changed the conserved histidine residue to a leucine. This change eliminated the ability of pN*bphOP1* to repress swarming (Fig. 12). Similar results were observed with a site-directed mutation using pN*bphP1* rather than pN*bphOP1* following introduction into Δ*bphP1* (1). These data establish that the histidine kinase activity of BphP1 is critical for the repression of swarming under white and red+far red light conditions.

BphP1-mediated repression of swarming was integrated with LOV-mediated activation of swarming

To evaluate the possibility of an interaction between BphP1 and LOV, Δ*lov*Δ*bphP1* and Δ*lov* Δ*bphP1*(pBphP1) were evaluated for differences in swarming under blue light. Δ*lov*Δ*bphP1* swarmed to wild-type levels (Fig. 13A), showing dominance of the Δ*bphP1* mutation over Δ*lov*. This dominance was confirmed by introducing pBphP1 into Δ*lov*Δ*bphP1*

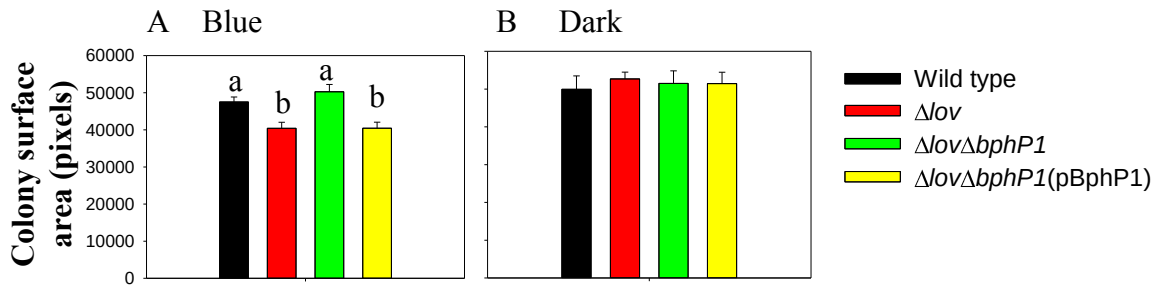

Figure 13. BphP1 repressed swarming under blue light as demonstrated by the reduction of swarming motility when *bphP1* is expressed in Δ*lov*Δ*bphP1*. Swarming was compared under (A) blue light and (B) in the dark. Values are as described in Fig. 10. This figure was published in collaboration with Liang Wu (1).

and observing a reduction in swarming to levels similar to Δ*lov* (Fig. 13A). Additional support for BphP1 repression under blue light was demonstrated by the decreased swarming motility of Δ*bphOP1*(pN*bphOP1*) compared the wild type under blue light (Fig. 12C). The observation that Δ*bphP1* and Δ*bphP1*(pBphP1) swarmed to similar levels under blue-light conditions (1), taken together with evidence of the role of BphP1 in blue-light sensing, demonstrate that BphP1 blue-light mediated repression of swarming requires the absence of LOV or overexpression of *bphOP1* (Figs. 12C and 13A). These results strongly support a model in which LOV activates swarming by reversing BphP1-mediated repression of swarming under blue light. Hyperswarming of Δ*bphOP1* and Δ*bphP1* still occurred under white light, demonstrating that LOV does not reverse red light-mediated repression by BphP1. Therefore, the BphP1-mediated response to red light occurs through a separate mechanism than its response to blue light.

BphP1 and LOV did not influence the rate at which cells swarm

Our previous collaborative efforts showed that Δ*lov* and Δ*bphP1* did not differ from the wild type in flagellar swimming or biosurfactant production (1), indicating that these were not the mechanisms by which BphP1 and LOV influence swarming, despite the fact that these both contribute to swarming in other organims (70). Here we evaluated if BphP1 and LOV influence

the rate at which *P. syringae* cells swarm. The wild type, Δ*lov*, and Δ*bphP1* were compared in their ability to traverse a strip of filter paper as described in Burch et. al (65). Δ*lov* and Δ*bphP1* did not differ from the wild type demonstrating that the altered swarming motility exhibited by these mutants was not attributable to changes in their rate of swarming (Fig. 14).

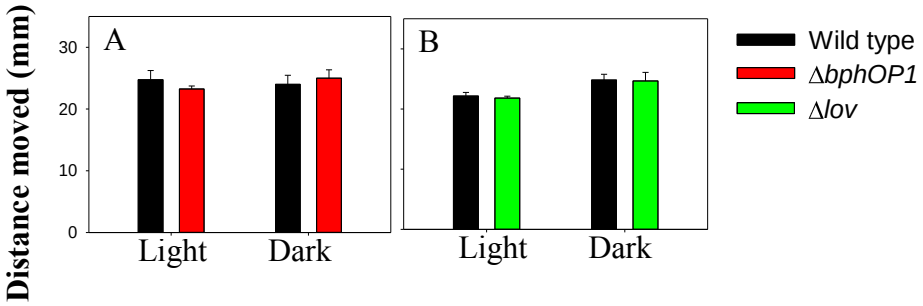

Figure 14. The Δ*bphOP1* and Δ*lov* mutants moved at the same rate as the wild type in both light and dark. The movement distance of (A) the wild-type and Δ*bphOP1* and (B) the wild type and Δ*lov* were compared by tracking their movement across a strip of filter paper. The values did not differ significantly ($P<0.05$, one-way ANOVA, $n=5$).

Discussion

In this work, we showed that light can be toxic to *P. syringa*e and reduce stationary-phase cell densities, but it can also serve as a cue that mediates the repression or attenuated repression of swarming motility. By evaluating the influence of distinct light wavelengths on the swarming motility of photosensory protein mutants, we demonstrated that the bacteriophytochrome BphP1 represses swarming through red- and blue-light sensing and integrates this repression with LOV-mediated attenuation of this repression. Our results are the first to demonstrate a physiological function for the bacteriophytochrome BphP1 in *P. syringae* and an integration of blue and red+far-red light signaling in bacteria. Additionally, this is the first evidence for a blue light-sensing bacteriophytochrome in bacteria.

BphP1 functions as a negative regulator of swarming motility in response to red light through its histidine kinase activity. This was supported by several lines of evidence. First, Δ*bphOP1* and Δ*bphP1* exhibited increased swarming motility when compared to the wild type under white (Figs. 8, 10A) and red+far-red (Fig. 10B, 12) light, and the increased colony surface area was due to increased spreading and not increased growth (Fig. 11). Second, overexpression of *bphOP1* in Δ*bphOP1* resulted in a swarm colony surface area smaller than the wild type. And third, BphP1 required its histidine kinase activity to negatively regulate swarming motility based on the loss of regulation of Δ*bphOP1*(pN*bphOP1H530L*), which expressed *bphOP1* but with a point mutation in the predicted autophosphorylation site.

BphP2, which has a unique tandem-histidine kinase domain structure, is not functionally redundant with BphP1 and does not have a role in swarming motility. Alignment of BphP1 and BphP2 demonstrated that the first 750 amino acids of BphP2 showed 51% identity with BphP1. However, the swarming motility of Δ*bphP1*Δ*bphP2* was much like that of Δ*bphP1* under all tested conditions suggesting that BphP2 does not have a role in swarming motility and that BphP1 and BphP2 are not functionally redundant (Fig. 10). BphP2 is unique among the three *P. syringae* photoreceptors in encoding two tandem histidine kinase domains. The first is a HisKA domain, similar to the HisKA domain in BphP1 and other bacteriophytochromes; however the second is classified as a HWE domain. HWE domains were first discovered in *A. tumefaciens* BphP2 and are distinguished from other histidine kinase domains by the absence of an F box and the presence of conserved H and W-E residues (71). BphP2 in both *A. tumefaciens* and *P. putida* have only a HWE domain, whereas the domain structure of *P. syringae* BphP2 is similar to *P. syringae* BphP1 with the addition of an HWE histidine kinase domain at the end of the protein. This additional histidine kinase domain was not included in the phylogenetic analysis because it

did not align to the other phytochrome sequences. It is possible that a recombination event between a newly duplicated *bphP1* gene and a gene encoding a HWE domain resulted in the presence of the tandem histidine kinase domains in BphP2, and that the acquisition of this new domain led to a loss of function.

LOV promotes swarming motility through blue light sensing. This conclusion is supported by the swarming motility of Δ*lov,* which was reduced in comparison to the wild type under white and blue light (Fig. 10). The difference in swarm colony surface area was not due to differences in cell number as demonstrated by the similar populations of the wild type, Δ*lov*, and Δ*lov*(pLOV) swarm colonies (Fig. 11). Loss of *bphP1* was phenotypically dominant to loss of *lov,* based on the result that the hyperswarming of Δ*bphP1*Δ*bphP2*Δ*lov* was much like that of Δ*bphP1* and Δ*bphP1*Δ*bphP2* despite the reduced swarming of Δ*lov* in white light (Fig. 10). Collectively, these results suggested that LOV promotes swarming motility upstream of BphP1-blue-light mediated repression.

BphP1 has blue-light sensing abilities and its blue-light-mediated regulation of swarming appeared to be integrated with LOV. This conclusion was supported by the increased repression of swarming motility in blue light when *bphOP1* was overexpressed in Δ*bphOP1* (Fig. 12). Additionally, the loss of *bphP1* was phenotypically dominant to the loss of *lov* in blue light, as shown by the swarming of Δ*bphP1*Δ*bphP2*Δ*lov* and Δ*lov*Δ*bphP1* as compared to Δ*lov* in blue light (Figs. 10 and 13). In all three cases where we observed BphP1 blue light-mediated repression of swarming, *bphP1* was overexpressed or LOV was absent. Blue light-mediated regulation by BphP1 was independent of red light-mediated repression based on that hyperswarming of Δ*bphOP1* and Δ*bphP1* still occurred under white light. Taken together these

results support a model where LOV promotes swarming by attenuating BphP1-mediated repression under blue light (Fig. 15).

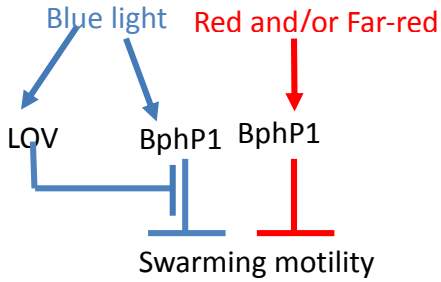

Figure 15. Model of the integrated signaling network formed by BphP1 and LOV to regulate swarming motility in response to red and/or far-red light and blue light in *P. syringae*. BphP1 represses swarming motility in response to red and/or far-red light and, through an independent pathway, represses swarming motility through blue light sensing; however, LOV positively regulates swarming by attenuating BphP1-mediated blue light sensing.

The ability of phytochromes to sense blue light may be conserved across evolutionary domains. Such blue light sensing is common in plants, with the phytochromes PhyA, PhyB, and PhyD all responding to blue light as well as red and/or far-red light (72). In addition, the cyanobacterial phytochrome Cph2 responds to blue as well as red light (34, 35). However, PhyA, PhyB, PhyD and Cph2 all interact with the chromophore phyocyanobilin (24), whereas bacteriophytochromes interact with biliverdin (13). It is noteworthy that in the case of Cph2 and BphP1 both blue-light mediated phenotypes are related to motility, suggesting that connection between blue light sensing and phytochromes may be conserved. The integration of a bacteriophytochrome and LOV signaling shows parallels to signaling by plant photoreceptors; specifically, blue light-mediated phytochrome regulation in *Arabidopsis thaliana* is repressed by the blue light-sensing cryptochromes CRY1 and CRY2 (72). The similarity between signal integration of BphP1 and LOV and these plant photoreceptors lends support for evolutionary conservation of integrated blue and red light-mediated signaling. This is the first example, however, of a LOV domain-containing protein interacting with a phytochrome.

LOV and BphP1 do not regulate swarming motility by altering flagellar swimming, biosurfactant production, or rate of swarming motility. In *P. syringae,* swarming requires active flagella and biosurfactant production. When flagellar activity and biosurfactant production were evaluated by visualization on agar plates (73), the wild type did not show differences between light and dark conditions, demonstrating that LOV and BphP1 regulate swarming independently of these traits (1). We showed that the wild type, Δ*lov,* and Δ*bphOP1* also did not differ in their rate of swarming across wet filter paper (65) under light and dark conditions (Fig. 14). Filter paper provides swimming channels through which the bacteria can traverse more rapidly than when in aqueous solutions (74), while providing an environment with heterogeneous water thickness where swimming is not always possible (65); these conditions are probably more representative of conditions on leaf surfaces than the homogeneous conditions on the surface of an agar medium.

The impact of light-mediated regulation of swarming on the *P. syringae* lifecycle remains to be determined. *P. syringae* survives in the soil in association with infected plant tissue and utilizes motility to colonize seedlings and eventually epiphytic and apoplastic leaf sites. Studies evaluating the spectrum of light that is transmitted through soil demonstrate that at a depth of as little as 3 mm much of the blue light is filtered out, whereas longer wavelengths are able to penetrate (75). Additionally, the ratio between red and far-red light changes depending on the depth of the soil (76). When in the soil, *P. syringae* may sense these changes in spectrum and alter its behavior. Similar filtration of blue light and changes in the red and far-red light ratio can occur as light passes through leaves, suggesting that *P. syringae* could use light sensing to determine its location on a leaf (77). Similarly, *P. syringae* could utilize the changes in light spectrum that occur throughout the day (78) to avoid circadian-regulated changes in plant

defenses (21, 22) in addition to anticipating the times of day with maximum potential for motility and infiltration through the stomata. Based on our results and experiments performed in *P. syringae* pv. tomato DC3000, pathovars may differ in their light-mediated regulation, particularly in the function of LOV. We demonstrated that LOV promotes swarming motility in B728a, whereas LOV represses swarming motility in DC3000 (44); however, these distinct behaviors could be attributable to differences in the environmental conditions of the assays if LOV responds to multiple environmental signals.

P. syringae showed reduced survival when stationary phase cells were exposed to light; however, BphP1, BphP2, and LOV were not involved in this photosensitivity. In particular, loss of *bphOP1*, *bphP2R*, or *lov* did not influence the reduction in density that occurred when cells were grown in white or blue light when compared to cells grown in the dark (Fig. 4, 5). We also demonstrated that the ability to adapt to a secondary stress, such as high osmolarity, was reduced when exposed to light (Fig. 6). Luria media had the largest effect on survival in stationary phase and it was also the medium with the lowest water potential (-1.4 MPa) (unpublished data, Kelly Peterson), providing further evidence that there may be a relationship between light and osmotic stress.

The photosensitivity of *P. syringae* was specifically in response to blue light based on that the reduced cell density in blue light was similar to that in white light. In *Porphyromonas gingivalis* and *Fusobacterium nucleatum,* photosensitivity resulted from the build-up of reactive oxygen species when exposed to wavelengths of light in the 400-500 nm range (79). Two common photosensitizers are porphyrin and flavin (80): heme is a type of porphyrin and is also the precursor for production of BV, which serves as the chromophore for BphP1 and BphP2, and the LOV chromophore is a flavin mononucleotide. Thus, the chromophores associated with

BphP1, BphP2, and LOV conceivably could have a role in *P. syringae* light sensitivity, although we did not observe a link between these two compounds and light sensitivity under the conditions tested.

Photoreceptors often regulate multiple pathways, indicating that BphP1 and LOV may regulate phenotypes beyond swarming motility. Photoreceptors in *Synechocystis* sp. strain PCC 6803 (33), *X. axonopodis* pv. citri (41), *Caulobacter crescentus* (46), and *R. leguminosarum* (45) all regulate multiple pathways. One approach to determine how BphP1 and LOV integrate their responses to light and identify other regulated physiological functions is to identify the downstream signaling components. Both BphP1 and LOV are orphan histidine kinases, meaning they are not encoded with their associated response regulators; this makes identifying downstream pathway components particularly challenging. Future studies will exploit swarming motility as a quantitative phenotypic assay in efforts to discover these pathway components.

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CHAPTER 3. *PSEUDOMONAS SYRINGAE* BACTERIOPHYTOCHROME BPHP1 CONTRIBUTES TO LEAF COLONIZATION AND REGULATES SWARMING INTIATION AND VIRULENCE VIA THE REGULATOR LSR

Abstract

Bacteriophytochromes are found in a range of heterotrophic bacteria, including those that live in association with plants, but little is known of the physiological processes they regulate. A recent report showed that a bacteriophytochrome encoded by *Pseudomonas syringae* pv. syringae B728a, BphP1, represses swarming motility through red- and blue-light sensing, and this repression is attenuated by a blue light-sensing LOV protein. In this work we demonstrate that a deletion mutant lacking the *bphP1* operon exhibited reduced survival on leaves in the 6 h after inoculation and increased population sizes by 48 h after inoculation, suggesting that BphP1 positively impacts short-term adaptation but negatively impacts longer-term adaptation to leaf surfaces. This mutant also exhibited increased movement from infected plant tissue to seeds in soil and increased lesion sizes on bean pods following stab inoculation, supporting a role for BphP1-mediated regulation of swarming motility in both movement from soil to seeds and lesion development. Collectively, these results are the first to demonstrate a role for a bacteriophytochrome in bacterial colonization and virulence of plants. Moreover, we further elucidate the role of BphP1 in swarming motility by demonstrating that loss of *bphP1* resulted in early swarming initiation suggesting that BphP1 represses the switch from a sessile to motile lifestyle. Additionally, we identify a regulator we designate Lsr as a component of the BphP1-LOV signal transduction pathway, supported by the altered swarming motility, swarming initiation, and lesion formation exhibited by a mutant lacking *lsr*. Furthermore, we provide evidence for the function of an acyl-homoserine lactone (AHL) signal molecule downstream of

Lsr in regulating swarming initiation, supported by the altered swarming initiation exhibited by mutants lacking *bphP1* and *lsr* with addition of purified AHL. Taken together, these results support a model where red light stimulates BphP1-Lsr-mediated activation of AHL production to regulate swarming motility.

Introduction

Pseudomonas syringae is a well-studied, foliar plant pathogen that causes disease on a wide range of economically important crops (1, 2). In addition, it is used as a model for understanding plant-bacterial interactions (3). *P. syringae* is a ubiquitous epiphyte that establishes large populations on the leaf surface before causing disease (4). During colonization *P. syringae* encounters a wide range of detrimental environmental conditions including ultraviolet light, oxidative and osmotic stresses, and nutrient-limited conditions (3). Although *P. syringae* is frequently studied in the context of the phyllosphere, it is also found in clouds, waterways, and snowpack (5). In all of these environments *P. syringae* utilizes a range of systems to adapt to environmental stresses including enzymes that quench reactive oxygen species, DNA repair systems (6), osmoprotectant accumulation (7, 8), and siderophore (9) and exopolysaccharide (10) production. *P. syringae* often encounters multiple stresses at once and the presence of one stress can serve as a cue to induce a variety of stress response pathways (11). Changes in the quality and quantity of light encountered in the phyllosphere and in the many other habitats *P. syringae* encounters may signal that that multiple environmental conditions are altered. For example, at mid-day, high levels of blue light exposure may be correlated with a high temperature and low water availability.

P. syringae is unusual among heterotrophic bacteria in that it encodes three photoreceptors, suggesting that light may serve as an important environmental signal.

Photoreceptors sense light through photosensory domains that interact with chromophores, which are excited by specific wavelengths of light (12). Chromophore excitation activate the interacting photosensory domain (12). Photoreceptors often include C-terminal output domains that initiate light-mediated responses (13). *P. syringae* encodes two bacteriophytochromes, BphP1 and BphP2, which respond to red/far-red light (14), and a blue-light sensing LOV protein (15). Our previous study of *P. syringae* pv. syringae B728a demonstrated that BphP1 responds to red/far-red and blue light and integrates its response with LOV to regulate swarming motility (16). Swarming motility in *P. syringae* is characterized by coordinated movement requiring biosurfactant production (17, 18) and functional flagella (19). Glycosylation of the flagella (20, 21), reconfiguration of the stators in the flagella motor (22-24), quorum sensing (25-27), and lipopolysaccharide components (28) all influence swarming motility among Pseudomonads. Other environmental conditions are known to influence swarming motility, such as high temperatures, which result in repression (29). Despite the mechanistic knowledge of swarming motility, the downstream components involved in light-mediated regulation of swarming motility have not been determined.

Beyond the characterization of the role of BphP1 in swarming motility in *P. syringae* (16), little is known about the physiological function of bacteriophytochromes in non-photosynthetic bacteria. Bacteriophytochromes have been studied *Deinococcus radiodurans* and *Pseudomonas aeruginosa* where they regulate pigment production (30) and heat tolerance and pyocanin production (31), respectively. LOV in *P. syringae* pv. tomato DC3000 represses virulence likely through blue-light mediated regulation of motility, quorum sensing, and attachment (32, 33). Red light increased the virulence of DC3000 (33), but the role of BphP1 in its colonization and virulence was not examined.

P. syringae BphP1 is composed of a chromophore-binding N-terminal domain and a C-terminal histidine kinase domain (14). BphP1 is autophosphorylated in response to red+far-red light (14), and its histidine kinase activity is critical for regulation of swarming motility in B728a (16). Histidine kinases function with response regulators as a mechanism for bacterial response to external stimuli; signaling occurs by transferring the phosphoryl group from an autophosphorylated histidine kinase to a conserved aspartate in the response regulator (34). Histidine kinases are often bifunctional with both kinase and phosphatase activities; the input stimuli of the histidine kinase can initiate phosphorylation or dephosphorylation of the interacting response regulator (35, 36). After receiving the phosphoryl group, the response regulator induces physiological changes in the cell by regulating gene expression, catalyzing reactions, or altering the activity of target proteins through direct interaction (37). Two-component systems are highly specific, and histidine kinases have a kinetic preference for their cognate response regulators both *in vitro* and *in vivo* (37). However, because multiple environmental stresses can occur at the same time, histidine kinases can also function in branched pathways where they interact with multiple response regulators, and numerous two-component systems communicate through cross-talk to coordinate their responses (38).

Identification of interacting downstream components can provide clues to how BphP1 regulates swarming based on the conserved functional domains of these components. Identification of interacting proteins is particularly difficult for BphP1 because it is not co-transcribed with its cognate response regulator. Instead *bphP1* is co-transcribed with *bphO*, which encodes the heme-oxygenase that provides biliverdin (14). Software using a Bayesian network method can identify characteristics of interacting protein domains through multiple sequence alignments and use these characteristics to predict protein-protein interactions (39).

These predictions have been used successfully to identify downstream components of the highly conserved histidine kinase, NblS, in the cyanobacteria *Synechococcus elongatus* PCC 7942 (40). These predictions were confirmed by yeast-two hybrid analysis, *in vitro* transfer of a phosphoryl group, and gene expression patterns (40). Application of this software to BphP1 may similarly identify interacting downstream components.

The objectives of this study were to (i) identify the mechanism of BphP1-mediated regulation of swarming motility, (ii) characterize the role of BphP1 in *P. syringae* plant colonization and virulence, and (iii) identify downstream components of the BphP1/LOV-mediated signaling pathway. The results show that BphP1 regulates swarming motility at least in part by regulating the initiation of swarming, suggesting regulatory control over a switch to active swarming. The results also show that BphP1 influences survival and growth on leaves, bacterial movement to a seed in the soil, and lesion development on bean pods, thus providing the first evidence of a bacteriophytochrome influencing bacterial colonization and virulence on plants. Lastly, the study identified a new regulator that functions downstream of BphP1 in regulating swarming motility and lesion development, but not plant colonization; because this regulator is encoded by a gene in the *lov* operon, it was designated Lsr for LOV-co-transcribed swarming repressor. The study elucidated the BphP1-signaling pathway further by demonstrating that Lsr functions upstream of an acyl-homoserine lactone (AHL) signal molecule; an AHL was previously shown to repress swarming (27). In sum, these results illustrate the influence of the bacteriophytochrome BphP1 on several phenotypes related to the interaction of *P. syringae* with its host and make inroads into understanding the signal transduction pathway involved in BphP1 regulation.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains and plasmids for this study are described in Table 1. *P. syringae* strains were grown in King's B medium (41) at 25°C unless otherwise described. *Escherichia coli* strains were grown in Luria medium at 37°C. Antibiotics were added at the following concentrations as needed (μg/ml): rifampin (Rif), 50; kanamycin (Km), 50; chloramphenicol (Cm), 30; cycloheximide (Cyclo), 100; and ampicillin (Amp), 50.

Table 1. Strains and plasmids used in this study

Strain or plasmid	Description of relevant genotype	Reference or source
***P. syringae* strains**		
B728a	Wild type; Rif^r	(42)
Δ*bphOP1*	B728a Δ*Psyr_3505-3504*; Rif^r	This study
Δ*lov*	B728a Δ*Psyr_2700*; Rif^r	This study
Δ*bphOP1*Δ*bphP2R*	B728a Δ*Psyr_3505-3504*Δ*Psyr_2385-2384*; Rif^r	This study
Δ*smpR*	B728a Δ*Psyr_2449*; Rif^r	This study
Δ*bphOP1*Δ*smpR*	B728a Δ*Psyr_3504*Δ*Psyr_2449*; Rif^r	This study
Δ*lov*Δ*smpR*	B728a Δ*Psyr_2700*Δ*Psyr_2449*; Rif^r	This study
Δ*lsr*	B728a Δ*Psyr_2699*; Rif^r	This study
Δ*bphOP1*Δ*lsr*	B728a Δ*Psyr_3505-3504*Δ*Psyr_2699*; Rif^r	This study
Δ*lov-lsr*	B728a Δ*Psyr_2700*-2699; Rif^r	This study
Δ*ahlR*	B728a Δ*Psyr_1622*::*km*; Rif^r Km^r	(43)
Δ*ahlIR*	B728a Δ*Psyr_1621-1622*::*km*; Rif^r Km^r	(43)
Δ*aefR*	B728a Δ*Psyr_3324::Tn5*; Rif^r Km^r	(43)
***E. coli* strains**		
NEB10β	Enables high-efficiency cloning	New England Biolabs Inc. MA
BL21-CodonPlus (DE3)-RILP	Enables protein expression under control of the T7 promoter with expression of rare *E. coli* codons; Spc^r Cm^r	Agilent Technologies, CA
Plasmids		
pME6041	Broad-host-range vector; Km^r	(44)

Table 1 continued

pN	pME6041 with *nptII* promoter next to a multiple cloning site; Km^r	(45)
pN*bphOP1*	pN with *bphO-bphP1* under the control of the *nptII* promoter in tandem with the *bphO* promoter; Km^r	(16)
pH	pME6041 with the high-expressing *Psyr_1321* promoter; Km^r	This study
pH*smpR*	pH with *Psyr_2449*; Km^r	This study
pH*lsr*	pH with *Psyr_2699*; Km^r	This study
pET21a	Vector for inducible expression of C-terminal His_6-tagged proteins; Ap^r	EMD Biosciences, Novagen
pET21a-*bphP1*	pET21a with *Psyr_3504*; Ap^r	This study
pET21a-*smpR*	pET21a with *Psyr_2449*; Ap^r	This study
pET21a-*smpR*D76A	pET21a-with a point mutation changing Asp to Ala at amino acid 76; Ap^r	This study
pET21a-*0886*	pET21a with *Psyr_0886*; Ap^r	This study
pET21a-*4376*	pET21a with *Psyr_4376*; Ap^r	This study
pET21a-*0489*	pET21a with *Psyr_0489*; Ap^r	This study
pET21a-*0488*	pET21a with *Psyr_0488*; Ap^r	This study
pET21a-*3433*	pET21a with *Psyr_3433*; Ap^r	This study
pET21a-*4392*	pET21a with *Psyr_4392*; Ap^r	This study
pET21a-*3299*	pET21a with *Psyr_3299*; Ap^r	This study

Construction of mutants and complementation constructs

Deletion mutants were generated by slice-overlap-extension PCR mutagenesis as described in Chapter 2. The single mutants referred to as Δ*lsr* and Δ*smpR* (Table 1), were used for construction of Δ*bphOP1*Δ*smpR*, Δ*lov*Δ*smpR*, and Δ*bphOP1*Δ*lsr* as described for double mutant construction in Chapter 2. The operon mutant Δ*lov-lsr* was constructed by amplifying the region upstream of *lov* and the region downstream of *lsr*; this construct resulted in loss of *lov*, *lsr*, and the region between these two genes. Primers utilized for the construction of each mutant can be found in Table 2. Δ*bphOP1*, Δ*lov*, and Δ*bphOP1*Δ*bphP2R* (16), and Δ*aefR*, Δ*ahlIR*, and Δ*ahlR* (43) were constructed as previously described.

To construct expression plasmids used to evaluate complementation of the Δ*lsr* and Δ*smpR* mutants, *lsr* and *smpR* were amplified by PCR and cloned into the EcoRV site of the pH vector, which was pME6041 with a 246-bp fragment containing the *Psyr_1321* promoter inserted upstream of the multiple cloning site. *Psyr_1321* was identified by microarray analysis to be a highly-expressed, constitutive promoter expressing a gene for a hypothetical protein; in particular, *Psyr_1321* was expressed at a level higher than 85% of the B728a genes in a basal medium and exhibited little change in expression in response to growth *in planta* or in a variety of stressful conditions (11). pN*bphOP1* was constructed as previously described (16).

Table 2. Primers used for this study

Primer[a]	Sequences[b]
Primers for constructing deletion mutants	
bphOP1-FL1-F	5'-GACAACGACGGTTTTCAGGTCAGC-3'
bphOP1-FL1-R	5'-AGCCTACACAATCGCTCAAGACGTGCTCTTGAGTGTGCAATGGGCAGA-3'
bphOP1-FL2-F	5'-AATATCCGGGTAGGCGCAATCACTCGCTTGCTCAGGCTTGCAGTG-3'
bphOP1-FL2-R	5'-GTGGCTACGCAAGTGCTGTTGG-3'
lov-FL1-F	5'-TCCGGGCCAGTATTGCGGCAATAG-3'
lov-FL1-R	5'-AGCCTACACAATCGCTCAAGACGTCATCCGTGTCGTTTCTACCGCCGC-3'
lov-FL2-F	5'-AATATCCGGGTAGGCGCAATCACTCGGCAATATGTATGACTTGCTGTTCAC-3'
lov-FL2-R	5'-CAGAATCGCACTCCAGGAGATTCG-3'
lsr-FL1-F	5'-TCGATGGTCTATGGCTTTGC-3'
lsr-FL1-R	5'-AGCCTACACAATCGCTCAAGACGTGCTGCATGAGTTTCGTGGTA-3'
lsr-FL2-F	5'-AATATCCGGGTAGGCGCAATCACTATTCTGCTGATCACGCTGGT-3'
lsr-FL2-R	5'-TACGATTATGTCGCCGTTGA-3'
smpR-FL1-F	5'-ATGCGCAGCACGGAAAAGACC-3'
smpR-FL1-R	5'-AGCCTACACAATCGCTCAAGACGTGGGAGGTTGCGCCAACCTGTG-3'
smpR-FL2-F	5'-AATATCCGGGTAGGCGCAATCACTTGGTAAATGAGCGGGCAACC-3'
smpR-FL2-R	5'-CTGCCGCCCTGACCCATC-3'
Kan cassette-F	5'-ATTGTGTAGGCTGGAGCTGCTTC-3'
Kan cassette-R	5'-CCATGGTCCATATGAATATCCTCC-3'
Primers for expressing *bphOP1*, *bphP1*, *bphP2R*, and *lov* in the deletion mutants	
bphOP1-F	5'-GCGGTGGCAAACCGTCGTCTTA-3'
bphOP1-R	5'-GCGCGAGGTACTTCCAGCGAAC-3'

Table 2 continued

lsr-F	5’-AAAGGACTATGACTATGATTCGCGA-3’
lsr-R	5’-TCAGACCGCAGCAACAACC-3’
smpR-F	5’-CACAGGTTGGCGCAACCTCCC-3’
smpR-R	5’-TCAAGCCAGCAGCTTCTGAA-3’
Primers for site-directed mutagenesis	
*smpR*DA-F	5’-GATGATTACCGCTTATCGGCTGC-3’
*smpR*DA-R	5’-GCAGCCGATAAGCGGTAATCATC-3’
Primers for construction of His_6-tagged proteins	
*bphP1*BamHIF	5’-GCCGGATCCATGAGCCAACTCGACAAAGACGCC-3’
*bphP1*NotII-R	5’-ATTTGCGGCCGCTCAAACCGCCATTGGCACCGTGAA-3’
*smpR*EcoRI-F	5’-GCGGAATTCATGCGTGCCGTGGTCGTAATGGCA-3’
*smpR*XhoI-R	5’ CCCCTCGAGAGCCAGCAGCTTCTGAATCTG -3’
*0886*EcoRI-F	5’-GCGGAATTCATGTCCACGCTTGCGCTATTGATATGC-3’
*0886*XhoI-R	5’-CCCCTCGAGGCTCATCATGTAGGCGAG-3’
*4376*EcoRI-F	5’-GCGGAATTCGTGGATAACTACCCGCTCACG-3’
*4376*HindIII-R	5’-CCCAAAGCTTGCCCTGGCTTTCGCTCTG-3’
*0489*EcoRI-F	5’-GCGGAATTCATGGCTCGAATATTGATCGTC-3’
*0489*XhoI-R	5’-CCCCTCGAGGCCGGCCAGCACCGCATTCAG-3’
*0488*EcoRI-F	5’-GCGGAATTCATGGAACAACACGCCTCCGCCCTG-3’
*0488*XhoI-R	5’-CCCCTCGAGTGATATGTGTTGTTCTGCTGC-3’
*3433*EcoRI-F	5’-GCGGAATTCATGGCAGTCAAGGTCCTGGTG-3’
*3433*XhoI-R	5’-CCCCTCGAGGAGACAAGCCTCTACCAG-3’
*4392*EcoRI-F	5’-GCGGAATTCATGAGCCAGAGCCTGAGCCAG-3’
*4392*XhoI-R	5’-CCCCTCGAGCCGGTTCCCCCACAGTCG-3’
*3299*EcoRI-F	5’-GCGGAATTCATGACCTGCAATCTGTTACTGGTCGAC-3’
*3299*HindIII-R	5’-CCCAAAGCTTATCGTCCAGACTGATAATCCG-3’
Primers for evaluating the *lov-lsr* operon structure	
*lovlsr*1-F	5’-AAGACGGCTCCTCGTTCTGG-3’
*lovlsr*1-R	5’-AGCACCATCTTCGCCAGTTC-3’
*lovlsr*2-F	5’-CGAAGGTCAAGGTGCTGTTG-3’
*lovlsr*2-R	5’-CCAACAGGTTCAGCAACAGC-3’
*lovlsr*3-F	5’-CGATACTGACAATGAACACATGAAG-3’
*lovlsr*3-R	5’ATTGGCAGTAAACGCACACA-3’
*lovlsr*4-F	5’-ACCGTATCGCCCCAGGTG-3’
*lovlsr*4-R	5’-TCAGACCGCAGCAACAACC-3’

[a] FL indicates flanking region

[b] underlined portions of the primer sequence indicate the region that binds to the kanamycin cassette

Assay for quantifying swarming motility

Analysis of swarming motility was performed as previously described (16) with a modified protocol for drying of swarm plates. Square petri plates (100 x 100 x 15 mm) containing 30 ml of King's B medium with 0.4% agar were either allowed to dry on the bench overnight or they were dried with the lids off in a laminar flow hood. For the latter, the bottom 8 in of the rear panel blower was covered to minimize spatial differences in air movement, and the plates were dried for 90 min. Strains were grown in King's B broth to late-log phase by shaking at 25^0C and were harvested, washed with nanopure water, and normalized to $4x10^8$ cell/ml by turbidity. Immediately after drying, the prepared plates were inoculated with 5 replicates of up to 5 strains, with each replicate comprised of a 2-µl drop containing approximately $8x10^5$ cells, using the plate design described in Wu et. al (16). The plates were closed with parafilm and were either subjected to light by placement under fluorescent bulbs (Ecolux F40C50-Eco, General Electric Co., Fairfield, CT) at a distance large enough to minimize altering the incubation temperature and such that the light intensity was 30 µmol $m^{-2}s^{-1}$, or were subjected to dark by enclosure in two layers of aluminum foil. If more statistical power was necessary to represent slight differences in surface area, at least two replicate plates were prepared for the light and dark treatments, and the four plates were incubated side-by-side at 22-23^0C for 10-14 h until swarming colonies were within no less than 5 mm to the nearest neighboring colony. After incubation, the plates were photographed and the surface area of each colony was quantified by recording the pixel counts using the selection tools in Adobe Photoshop, as previously described (16). Analysis of variance (ANOVA) was performed on the pixel counts for the strains on a single representative plate (n = 5), or if multiple plates were necessary to demonstrate

differences, a two-way ANOVA was performed comparing across strains and plates. Each strain comparison was performed a minimum of three times.

Assay for quantifying swarming initiation

P. syringae cells inoculated onto swarm plates exhibited a circular region of confluent growth prior to the appearance of bulges that eventually developed into outward-radiating tendrils indicative of swarming motility. To identify the time at which swarming motility was initiated following inoculation, swarm plates and inocula were prepared as described above, and the inocula were concentrated 5-fold by centrifugation prior to placement on the plates. The use of higher-density inocula reduced the time between inoculation and swarm initiation from 8-10 h to 3-5 h. After inoculation, the cell density in the residual inoculum suspensions were quantified by viable plate counts to validate similar inocula concentrations. Plates were inoculated and incubated under light and dark conditions, as described above, as well as under white light, red+far-red light (680-750 nm), and blue light (470 nm). Bilirubin bulbs (Interlectric Biliblue, Warren. Pennsylvania) were used for blue light treatments, with the cells exposed at a distance such that the light intensity was 21 µmol $m^{-2}s^{-1}$. F40T12R bulbs (Interlectric Corp, Warren, Pennsylvania) were used for red+far-red light treatments at a distance that the light intensity was 6.5 µmol $m^{-2}s^{-1}$. Each strain was randomly assigned a code and that code was used for the duration of the experiment so that their identities were unknown during evaluation. The plates were examined at 0.25-0.5-hour intervals after the initial 2-3 h of incubation, and the time at which each strain exhibited detectable tendril initiation was recorded. For each strain at each time point, the proportion of colonies exhibiting detectable tendril initiation on each of four plates was averaged and the strains were compared using ANOVA on arcsine normalized values. To determine the role of an acyl-homoserine lactone signal molecule in swarming initiation, the

inoculum cells were amended with purified N-(β-ketocaproyl)-L-homoserine lactone (Sigma Aldrich Corp. St. Louis, MO) to a final concentration of 0.1 µM immediately before placement on the plates. Each comparison was performed a minimum of three times.

Quantification of bacterial movement in soil from infected leaf tissues to seeds

P. syringae movement from infected plant debris in the soil to newly planted bean seeds was quantified by generating infected leaf tissue, mixing this into soil that was then introduced into the field, introducing seeds into the inoculated soil, and enumerating the bacteria on the seeds after various periods of incubation. Infected leaf tissue (*P. phaseolus* cv. Bush Blue Lake 274) was generated by inoculating two-week-old bean plants by dip inoculation into a liter of PB buffer containing bacteria suspended at a concentration of $4x10^6$ cells/ml. Plants were then incubated at room temperature under 12 h-light/dark cycles using white fluorescent lights until disease symptoms developed (10 days), at which time leaves were collected and homogenized; homogenization was carried out by cutting the leaves into squares (approximately 1 x 1 mm^2) and CFU/g of wet leaf tissue was determined. Soil was collected from the field site at the ISU Horticulture Research Station, and for each strain, the wild type, Δ*bphOP1*, or Δ*bphOP1*Δ*bphP2R,* approximately 6.5 kg of soil was amended in the lab with the infected leaf tissue to a final density of approximately $1x10^5$ cells/g of soil by evenly mixing the homogenized leaf tissue into the soil. The soil was covered and kept at room temperature overnight allowing the bacteria time to adapt to the soil conditions. Following overnight incubation five 1-gram samples were collected to confirm the homogenous distribution of infected plant tissue and to determine bacterial density. In the field, inoculated soil was introduced into 2.5 x 5-cm holes (WxD) and one seed (cv. Bush Blue Lake 274) was planted at a depth of 1 cm along with 5 ml of sterile water. A block design was used in which each strain was represented in four 1 x 0.23-m

plots that were spaced 0.6 m apart. Each plot contained 20 seeds, which were placed approximately 5 cm apart. To estimate *P. syringae* growth on seeds in the absence of movement, a treatment was included in which seeds that had been placed in soil containing plant tissues infected with the wild type were removed after approximately 1 h and were planted in holes containing soil that had not been amended with infected leaf tissue. At each time point 4 seeds were collected per plot for each strain for a total of 16 seeds per strain. Each seed was sonicated for 7 min in washing buffer and *P. syringae* populations were enumerated on King's B medium containing Rif and Cyclo. The populations were expressed as CFU/seed and were log-transformed before analysis, with the samples at time 0 that were below the detection limit estimated as Log(0.5 CFU/seed). Differences in seed populations among strains at each time point were evaluated using ANOVA.

Assay for quantifying lesion development on bean pods

Virulence was assessed based on the size of lesions developed following inoculation with bacteria into bean pods. *Phaseolus vulgaris* bean pods were obtained from the local grocery store and were sterilized by soaking in 2% sodium hypochlorite for 10 min and rinsed in deionized water. Bean pods were placed on top of filter paper that was soaked in 50 ml of sterile nanopure water in a 23 x 33 cm glass tray. Each bean pod was inoculated with a culture of each of the strains compared in an experiment, and sufficient bean pods were used to examine at least three independent cultures of each strain and at least two replicate bean pods for each comparison. The bean pods were pierced with a plastic pipette tip to a depth of no more than 2 mm. A 2-µl suspension containing $8x10^5$ cells, which was prepared as described for the swarming assay, was introduced gently into the resulting hole. The glass tray was covered with plastic wrap and placed under white light. The bean pods were incubated for 36-48 h at 22-23^{0}C and then

photographed. The surface area of each water-soaked lesion was quantified based on pixel counts as described for swarming. ANOVA was performed to evaluate differences in the lesion sizes among strains, and experiments for each comparison were replicated a minimum of three times.

Bacterial enumeration on leaves

Bacterial colonization of plant leaves was evaluated using inocula containing cells collected from swarm plates. These inocula were prepared by growing and inoculating cells as in the swarming assay described above, except that a single 2-µl drop containing approximately $8x10^5$ cells was placed in the center of each of three replicated plates for each strain. The plates were incubated in the dark at 22-23^{0}C for 16 h after which the bacteria had swarmed over the majority of the plate. Following incubation and visual confirmation of extensive swarming, cells from the whole plate were resuspended in 1.5 ml of sterile nanopure water and the cultures were normalized to $4x10^8$ cells/ml based on turbidity. The three replicates for each strain were then pooled and diluted to a concentration of $4x10^6$ cells/ml in 1 liter of sterile nanopure water. At least five pots containing 8-10 two-week-old bean plants (*P. vulgaris* cultivar Bush Blue Lake 274) were inoculated by leaf immersion in the bacterial suspension for 30 sec. After inoculation plants were incubated in a growth chamber (Conviron, Winnipeg, Canada) set to 90% relative humidity with a 12h light/dark photoperiod. Because motility of B728a is repressed above 25^{0}C (29) plants were kept at approximately 20^{0}C by altering the programmed lighting to compensate for light-generated heat in the daytime. For each time point 5 to 12 leaves per strain were collected and four 1.3-cm diameter circular discs were collected from each leaf. The discs from a single leaf were combined and homogenized in 300 µl of 10 mm phosphate buffer (pH 7) (PB), and bacterial populations were enumerated on King's B medium containing Rif and Cyclo. ANOVA was performed on the log-normal values to evaluate differences in bacterial populations

among strains at each time point, and a repeated measures analysis of the log-normal values was performed using a split-plot design where strain was the whole-plot factor and time was the split-plot factor, with subsampling within the split plot using Proc Glimmix in SAS. Experiments for each comparison were performed a minimum of three times.

Bacterial colonization was also examined on leaves under field conditions at the Iowa State University Horticulture Research Farm near Gilbert, Iowa. Bush Blue Lake beans were grown in a block design with four 1 x 0.5-m blocks per strain treatment and approximately 40 plants per block. Strains were recovered from King's B medium containing 1.5% agar after a 48-h incubation, and a suspension of $4x10^7$ cells/ml in PB was inoculated onto the leaves of two-week-old bean plants by application with a hand sprayer. At 0, 3, 6, and 10 days after inoculation, 4 leaves were collected from each block. Bacteria were recovered by sonication for 7 min in 20 ml of washing buffer (PB and 5 g/liter proteose peptone) and enumerated on King's B medium containing Rif and Cyclo. Differences between wild-type and mutant strains were calculated using a Student's *t*-test.

Identification and analysis of candidate BphP1-interacting proteins

Response regulators that may interact with BphP1 were selected using Prediction of Interaction Specificity of Two-component Systems software (Swiss Institute of Bioinformatics) (39). Tagged derivatives of BphP1 and eight of these putative BphP1-interacting proteins, Psyr_2449 (SmpR), Psyr_0886, Psyr_4376, Psyr_0489, Psyr_0488, Psyr_3433, Psyr_4392, and Psyr_3299, were generated by inserting the gene into the multiple cloning site of pET21a to create a C-terminal His_6-tagged fusion (Table 1). Site-directed mutagenesis of pET21-*smpR* was performed using a QuikChange II XL site-directed mutagenesis kit resulting in the construct pET21-*smpR*D76A. The constructs were introduced into the protein expression strain

BL21(DE3) Codon-plus-RILP (Agilent Technologies, Santa Clara, CA). Following growth of the cells to late-log phase and induction with 10 mM IPTG at 20°C for 16 h, His_6-tagged proteins were purified using a PerfectPro Ni-NTA Agarose nickel affinity column (5 PRIME Inc, Gaithersburg, MD).

A phosphoryltransfer assay was performed to evaluate BphP1 phosphorylation of candidate response regulators. BphP1 was first autophosphorylated by incubating 10 µl of purified BphP1 (1 mg/ml, 12.18 µM) under red light (680 nm, 10 µmol $m^{-2}s^{-1}$) using light-emitting diodes (Marubeni America Corporation, New York, NY) in the presence of biliverdin (Frontier Scientific, Logan, UT) provided at a ten-fold molar excess. After 10 min, 10 µl of reaction buffer was added that contained the following: 50 mM Tris/HCl (pH 7.6), 4 mM $MgCl_2$, 100 mM KCl, 0.4 mM EDTA, 2 mM dithiothreotol, 0.04 mM ATP (non-radioactive), and 0.2 µM [γ-^{32}P] ATP. Samples were incubated for 5 min, amended with an equimolar concentration of a purified candidate response regulator, incubated for 5 min, and amended with 20 µl of sample buffer containing 62.5 mM Tris/HCl (pH 6.8), 25% glycerol, 2% SDS, 0.01% bromophenol blue, and 5% β-mercaptoethanol. The protein mixtures were subjected to SDS-PAGE using Novex 12% Tris-Glycine Midi gels (Life Technologies, Grand Island, NY) and fixed by washing in 40% methanol and 7% acetic acid for 15 min. The gel was then subjected to phosphorimaging (PharosFX Plus Molecular Imager, Bio-rad, Hercules, CA) to detect radioactive species, and then was stained with Coomassie blue for visualization of proteins. The radioactivity in the gel bands was measured in counts per minute using a liquid scintillation counter (Tri-carb 2100TR, Packard-A Packard BioScience Company, Meriden, CT).

Characterization of the *lov-lsr* operon structure

P. syringae cells were grown overnight in King's B media and sub-cultured into fresh King's B media prior to four hours of additional growth in the dark. RNA was then extracted using the RNeasy Mini Kit (Qiagen, Venolo, Limburg), and contaminating DNA was removed using on-column DNase I digestion. Using primers designed for *lov*, *lsr*, and the *lov-lsr* intergenic region (Table 2), cDNA was amplified using the SuperScript III First-Strand Synthesis System kit (Invitrogen, Carlsbad, CA). Amplified fragments were separated and visualized using gel electrophoresis.

Results

BphP1 negatively regulated swarming initiation in response to white and red light

P. syringae BphP1 was previously shown to negatively regulate swarming motility based on the hyperswarming of a *bphP1* deletion mutant compared to the wild type when exposed to white and red light (16). In those studies, we observed that swarm colonies exposed to red and white light not only had smaller surface areas but also began to form tendrils later than colonies kept in the dark. Here, we identified the time at which this swarming initiation occurred based on the appearance of a bulge along an otherwise smooth colony edge. To evaluate if BphP1 was involved in light-mediated repression of swarming initiation, Δ*bphOP1* and Δ*bphOP1*(pN*bphOP1*) were constructed (Fig. 1) and inoculated onto soft agar plates, and the

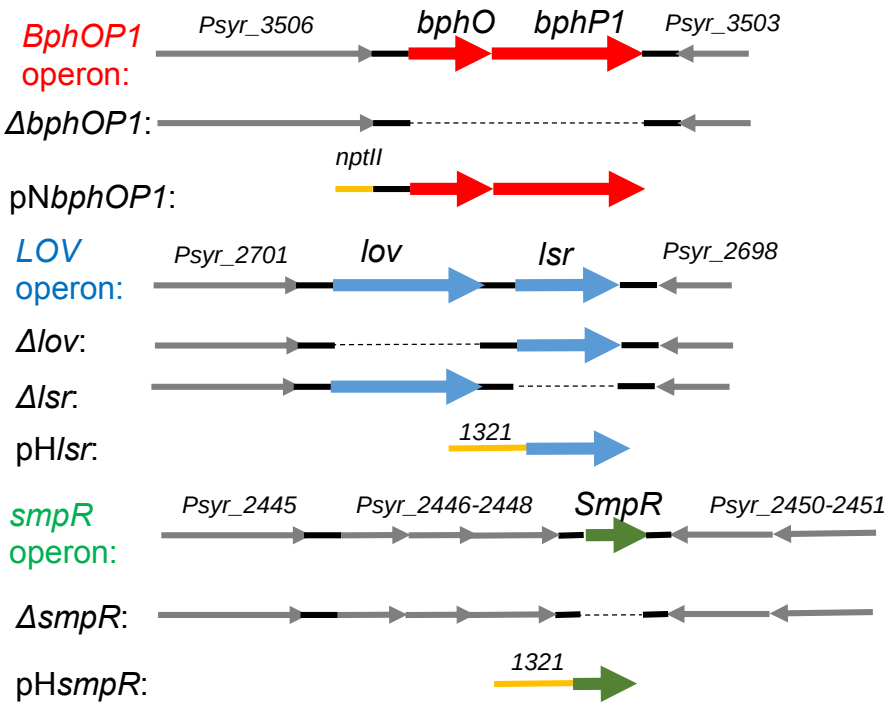

Figure 1. *bphOP1* and *Psyr_2445-2449* are predicted to be polycistronic and *lov* and *lsr* are predicted to be monocistronic (www.pseudomonas.com). Below each operon is a list of the deletions and the constructs used to test for complementation of the phenotypes of interest. The constitutive promoters in the pN and pH vectors were derived from *nptII* and *Psyr_1321*, respectively.

time at which tendril formation was first detected was recorded for each colony. In white light Δ*bphOP1* initiated tendril formation earlier than the wild type based on the larger proportion of colonies forming tendrils at 3.25 and 3.5 h after inoculation (Fig. 2A). Similarly, in red light significantly more colonies of Δ*bphOP1* formed their first tendrils at 3. 5 and 3.75 h after inoculation than the wild type (Fig. 2B); however, in blue light Δ*bphOP1* (Fig. 2C) and the wild type did not differ significantly in tendril formation. Collectively, these results shows that BphP1 represses swarming initiation in response to white and red light but not blue light. Expressing *bphOP1* under the control of a constitutive promoter, *nptII*, in tandem with the *bphOP1* native promoter on pN*bphOP1* resulted in greater expression of *bphOP1* than with the *bphOP1* promoter alone (16). When pN*bphOP1* containing this construct was introduced into Δ*bphOP1*,

tendril formation was similar to the wild type under all tested conditions (Fig. 2A-C). In summary these results demonstrate that BphP1 represses swarming initiation in response to red and white but not blue light.

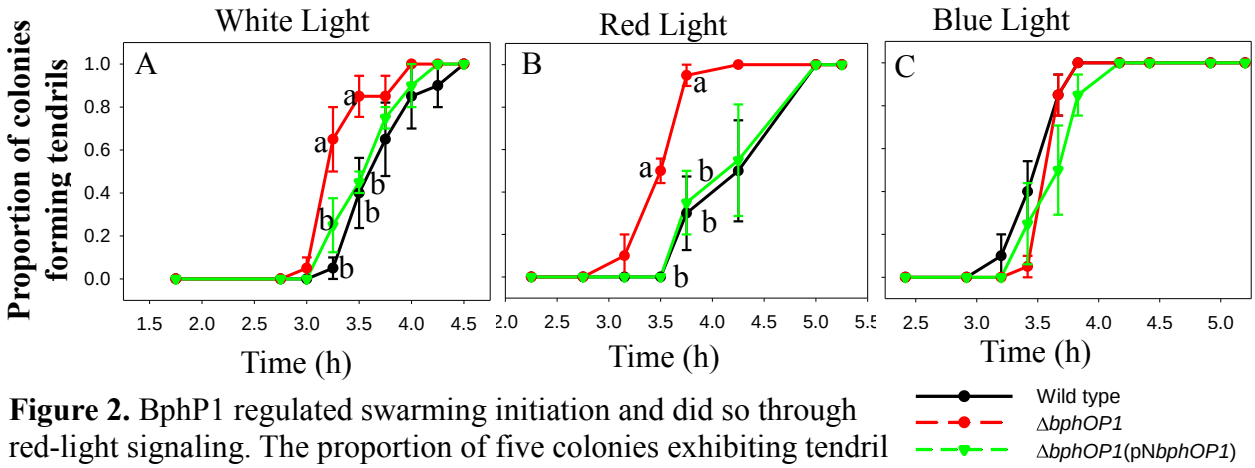

Figure 2. BphP1 regulated swarming initiation and did so through red-light signaling. The proportion of five colonies exhibiting tendril formation was evaluated in the wild type, Δ*bphOP1*, and Δ*bphOP1(*p*NbphOP1*) in (A) white, (B) red, and (C) blue light. Values in the same figure indicated by the same letter do not differ significantly for comparisons within a single time point ($P<0.05$, one-way ANOVA of arcsine transformed data, n=4). Error bars represent the standard error.

BphP1 negatively regulated movement to seeds and lesion development on *Phaseolus vulgaris* bean pods

To determine if BphP1 repression of swarming motility influences the ability of *P. syringae* to move to seeds, we introduced wild type- and Δ*bphOP1*-infected plant tissues into soil to mimic a natural inoculum source and monitored bacterial movement to seeds. To differentiate between growth and movement, we incubated one set of seeds for 1 h in the soil inoculated with the wild type and transferred them to un-inoculated soil, thus enabling the growth of the initial inoculum to be monitored in the absence of subsequent movement to the seed. The bacterial populations on seeds were generally significantly higher by 72 and 96 h after inoculation in soil that contained wild type- or Δ*bphOP1*-infected leaf tissues than on seeds that

had been planted and transferred to the un-inoculated soil, designated "transferred seeds" (Fig. 3), indicating that *P. syringae* was able to move from the infected leaf tissues to the seeds in soil. Δ*bphOP1* established significantly higher populations by 24, 48 and 72 h than the wild type, demonstrating that BphP1 represses this movement. This experiment was performed twice in the field with Δ*bphOP1*, and although the growth dynamics differed between the experiments due to variation in environmental conditions, Δ*bphOP1* was present in larger populations on seeds in both experiments (Fig. 3A, B). This experiment was also performed with Δ*bphOP1*Δ*bphP2R*, the wild type, and a "transferred seed" treatment (Fig. 3C). Δ*bphOP1*Δ*bphP2R* also established significantly higher populations than the wild type and the control by 24 h. BphP2 was previously demonstrated to not have a role in swarming motility (16), suggesting that the difference in population was due to the loss of *bphOP1*. Taken together these results support a role for BphP1 in repressing movement within the soil.

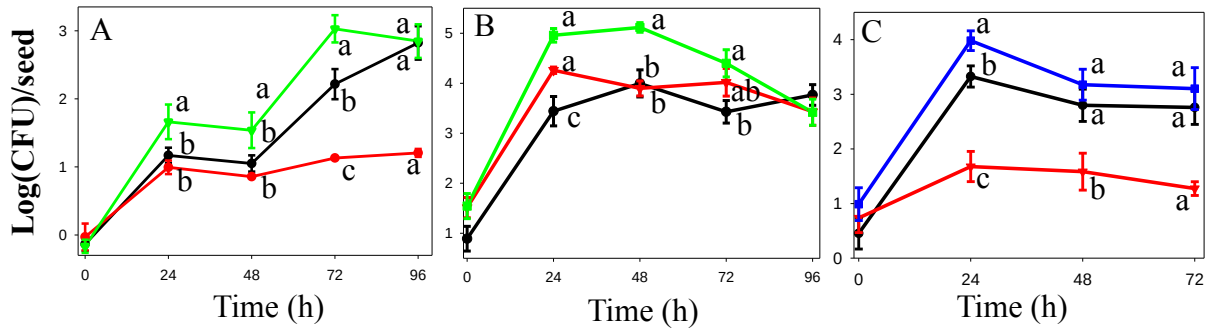

Figure 3. BphP1 repressed movement from the infected leaf tissue in the soil to seeds. The populations of the wild type, Δ*bphOP1,* and Δ*bphOP1*Δ*bphP2R* were measured at various times on seeds after planting in soils containing leaf tissues infected with each strain, or after incubating in soil with leaf tissue infected with the wild type and transferring to un-inoculated soil, designated "transferred seeds". Values and error bars are as described in Fig. 2 and comparisons were made within time points (P<0.05, one-way ANOVA, n=16).

To investigate if BphP1 and LOV contribute to lesion formation, we inoculated bean pods with the wild type, Δ*lov*, Δ*bphOP1*, and Δ*bphOP1*(pN*bphOP1*) strains by injection as previously described (46) and incubated them in either light or dark conditions for 36-48 h. When exposed to light, Δ*bphOP1* formed significantly larger water-soaked lesions (Fig. 4). In contrast, Δ*lov* consistently formed smaller water-soaked lesions in multiple experiments, although these differences were not significant. These results demonstrate that BphP1 and possibly LOV regulate virulence on bean pods and do so in a manner analogous to their regulation of swarming motility. The lesions induced by the wild type and Δ*bphOP1* did not differ significantly in size in the dark conditions (Fig. 4A), demonstrating that BphP1 regulates virulence on bean pods in response to light. Complementation of the Δ*bphOP1* mutant restored lesion formation to wild-type levels (Fig. 4B), confirming a role for BphP1 in repressing lesion formation.

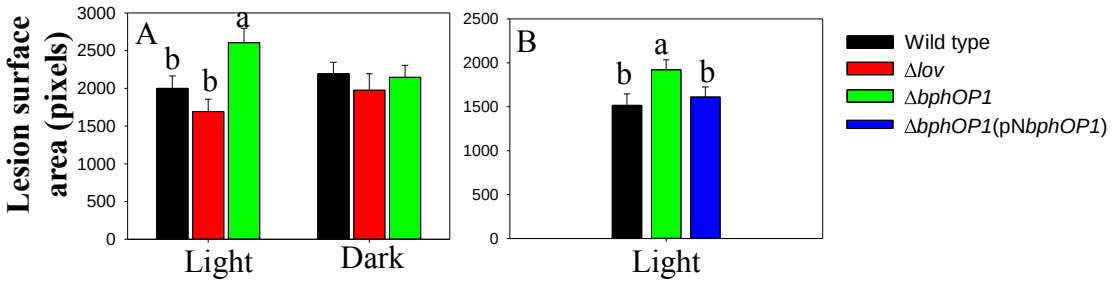

Figure 4. BphP1 repressed lesion development in the light but not in the dark. Lesion sizes of the wild type, Δ*lov*, Δ*bphOP1,* and Δ*bphOP1*(pN*bphOP1)* were compared in either (A,B) white light or (A) dark conditions. The lesion sizes were quantified based on pixel counts using Adobe Photoshop. Values and error bars are as described in Fig. 2 (A, $P<0.05$, two-way ANOVA based on strain and experiment, n=9; B, $P<0.05$, one-way ANOVA, n=8).

BphP1 contributed to *P. vulgaris* colonization

Motility is important for leaf colonization (47). To evaluate if BphP1 contributes to leaf colonization, the wild type, Δ*bphOP1*, and Δ*bphOP1*(pN*bphOP1*) were inoculated onto *P.*

vulgaris by leaf submersion, and total populations were determined based on viable plate counts of homogenized plant tissue. Δ*bphOP1* exhibited significantly reduced survival on leaves in the 6 h following inoculation ($P<0.05$) but established populations that were slightly larger than those of the wild type by 48 h (Fig. 5). Additionally, Δ*bphOP1*(pN*bphOP1*), which contains *bphOP1* expressed under the control of a tandem *nptII-bphOP1* promoter, showed inverse behavior to Δ*bphOP1,* with significantly higher populations by 6 h ($P<0.05$) but smaller populations by 48 h (Fig. 5A). These trends were observed in three replicate experiments, and the results in Fig. 5A are representative of the three experiments. The log-normal values of these three experiments were pooled and analyzed using a linear mixed effects model where strain was the whole-plot factor and time was the split-plot factor, with subsampling within the split plot. This analysis determined that Δ*bphOP1* differed significantly from the wild type ($P=0.03$) and fromΔ*bphOP1*(pN*bphOP1*) ($P=0.0005$) at 48 h. The growth trends of reduced population sizes following inoculation and subsequent recovery to wild-type levels were also observed in studies conducted in the field (Fig. 5B). These findings indicate that BphP1 positively contributes to survival on leaves in the early stages of colonization but may negatively regulate leaf colonization at later stages.

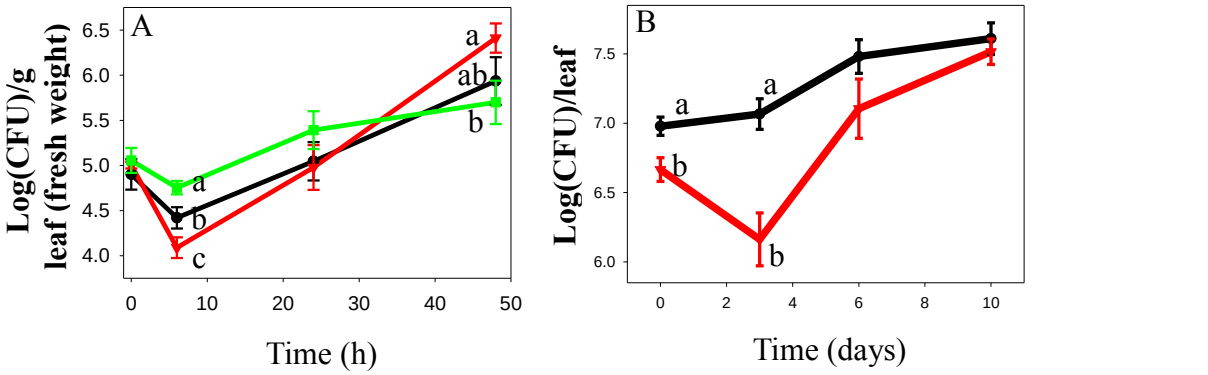

Figure 5. BphP1 positively contributed to survival in early stages of colonization (<10 h) and negatively regulated colonization in later stages (>24 h). Population sizes of wild type, Δ*bphOP1*, and Δ*bphOP1*(pN*bphOP1)* on leaves in (A) a growth chamber and (B) the field were compared. Bacteria were recovered from leaves by homogenization. Values and error bars are as described in Fig. 2 and comparisons were made within a time point (A, P<0.05 at 6 h; P<0.1 at 48 h, comparisons were based on a one-way ANOVA, n=12; B, P<0.05 in a Student's *t*-test).

Psyr_2449 (SmpR), a putative BphP1-interacting protein, was phosphorylated by BphP1 *in vitro*

Software (39) was utilized to identify candidate response regulators with the highest potential for interaction with BphP1; this software was designed to predict protein-protein interactions in two-component systems through a Bayesian algorithm formulated by evaluating the amino acid composition of known two-component systems . The software identified eight proteins with a probability of interaction above 0.001 (Table 3). For further analysis we focused on the three with the highest probability of interaction: Psyr_2449, Psyr_0886, and Psyr_4376, in addition to two response regulators predicted to function in motility: Psyr_0489 and Psyr_0488. As an initial test of the validity of the predicted interactions, a yeast-two hybrid system was utilized and the results supported possible interactions between the predicted interacting response regulators and BphP1 (Appendix A).

Table 3. Proteins predicted to interact with BphP1

Proteins	Probability of interaction	Predicted domain structure and function
Psyr_2449 (SmpR)	0.596	CheY-like REC domain, encoded with chemotaxis genes
Psyr_0886	0.181	CheY-like REC domain with CheC domain
Psyr_4376	0.138	CheY-like REC domain, LuxR-like DNA binding domain
Psyr_0258	0.053	OmpR, osmolarity response regulator
Psyr_0489	0.017	CheY-like REC domain, PilH ortholog involved in twitching motility in other Pseudomonads
Psyr_5032	0.007	CheY-like REC domain, DNA binding effector domain
Psyr_3091	0.004	CheY-like REC domain, DNA binding effector domain
Psyr_0488	0.004	CheY-like REC domain, PilG ortholog involved in twitching motility in other Pseudomonads

In *P. syringae* pv. tomato DC3000, BphP1 is autophosphorylated by red light in the presence of biliverdin (14). To confirm that BphP1 in B728a is similarly activated by red light when incubated with biliverdin, purified BphP1 was incubated with ^{32}P in the presence or absence of biliverdin while exposed to red light (680nm) or dark conditions (Fig. 6A). Because BphP1 exhibits some blue light-mediated repression of swarming, purified BphP1 was also tested for phosphorylation under blue light (470nm), but autophosphorylation was not observed (Fig. 6A). These results confirm that the B728a BphP1 requires biliverdin for activation and is autophosphorylated in response to red but not blue light.

Two component signal transduction requires the transfer of a phosphoryl group from the conserved residue of a histidine kinase to a conserved aspartate in the receiver domain of an interacting response regulator, and usually occurs with some degree of specificity (38). To evaluate if any of the response regulators were phosphorylated by BphP1, purified BphP1 was exposed to red light in the presence of ^{32}P and then co-incubated with either Psyr_2449,

Psyr_0886, Psyr_4376, Psyr_0489, or Psyr_0488 (Fig. 6B). Due to the possibility of cross-talk between BphP1 and LOV response regulators, the potential LOV-interacting proteins Psyr_3433, Psyr_4392, and Psyr_3299 were also tested for interactions with BphP1. Only one of these proteins, Psyr_2449, was clearly phosphorylated, demonstrating that it interacted with BphP1 *in*

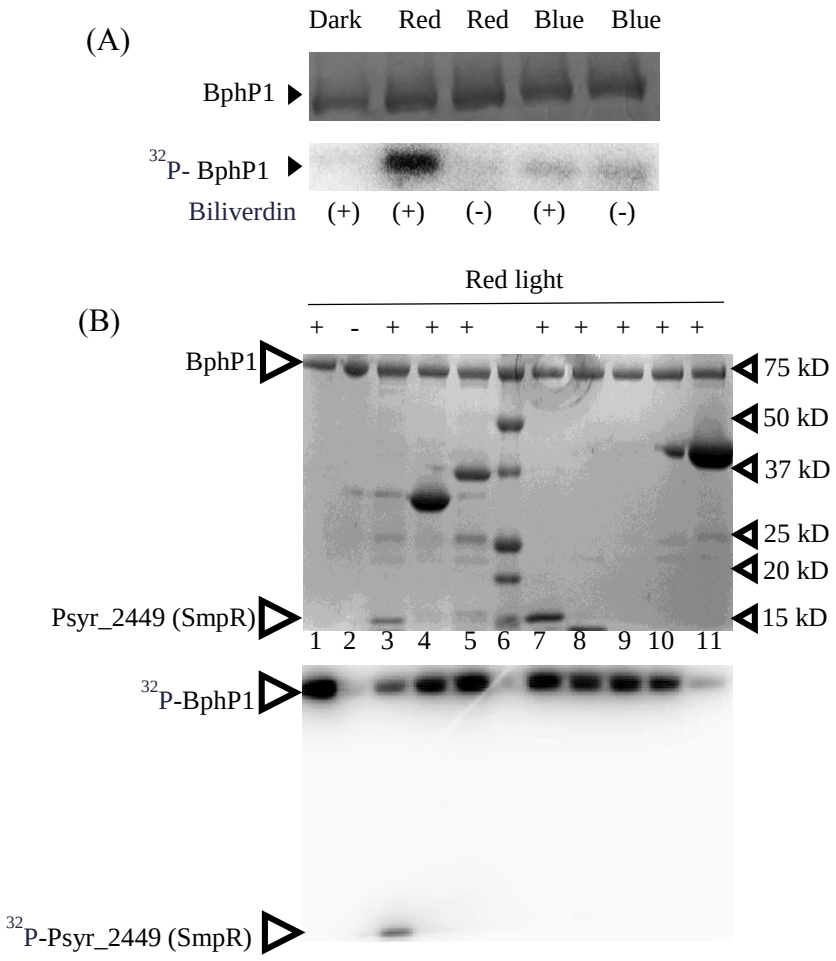

Figure 6. BphP1 was autophosphorylated when exposed to red but not blue light and it transferred its phosphoryl group to Psyr_2449. (A) Purified BphP1 was incubated with ^{32}P in the presence (+) or absence (-) of biliverdin in red and blue light. (A, B) Following light exposure purified proteins were subjected to SDS-PAGE and exposed to the phosphoimaging. (B) Samples were incubated with BphP1 in the presence of ^{32}P in the presence (+) or absence (-) of red light, as indicated. By lane in order from left to right: (1) autophosphorylated BphP1 exposed to red light, (2) BphP1 kept in the dark, and autophosphorylated BphP1 incubated with (3) Psyr_2449, (4) Psyr_4376, (5) Psyr_3433, (6) protein standards, (7) Psyr_0488, (8) Psyr_0489, (9) Psyr_4392, (10) Psyr_3299, and (11) Psyr_0886.

vitro. By measuring the extent of phosphorylation of Psyr_2449 by BphP1 over time based on band intensity (Fig. 7A) and counts per minute in the band (Fig. 7B), we determined that the

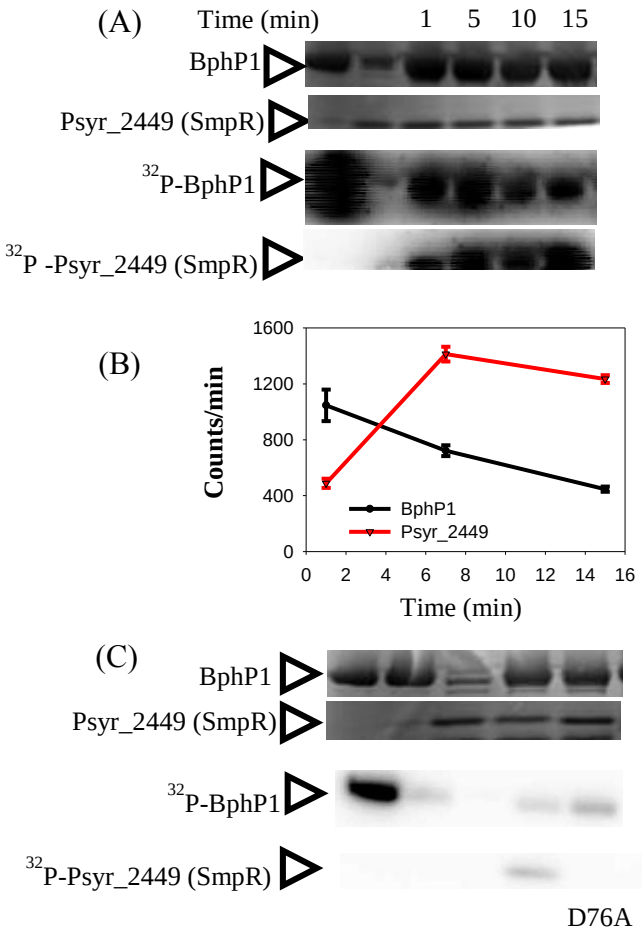

Figure 7. SmpR was phosphorylated by BphP1 and this phosphorylation required a conserved aspartate residue in SmpR. (A) The transfer of ^{32}P from BphP1 to SmpR increased over time. By lane in order from left to right: BphP1 autophosphorylated in response to red light, SmpR incubated in the absence of autophosphorylated BphP1 for 15 min, and autophosphorylated BphP1 and SmpR co-incubated for 1, 5, 10 and 15 min. (B) The transfer of ^{32}P from BphP1 to SmpR over time was quantified based on counts per minute, where BphP1 and SmpR were co-incubated for 1, 7 and 15 min and the resulting gel (not shown) was subjected to phosphoimaging. (C) Loss of a conserved aspartate in SmpR was associated with loss of phosphorylation. By lane in order from left to right: BphP1 autophosphorylated in response to red light, BphP1 incubated in the dark, SmpR incubated in the absence of BphP1, autophosphorylated BphP1 co-incubated with SmpR for 5 min, and autophosphorylated BphP1 co-incubated with SmpRD76A for 5 min. All samples were incubated in the presence of ^{32}P.

radioactivity of BphP1 decreased and the radioactivity of Psyr_2449 increased over time. Psyr_2449 was also incubated in the presence of ^{32}P and in the absence of BphP1 (Fig. 7A) and it did not autophosphorylate, supporting that BphP1 was the source of the observed phosphorylation. To evaluate the role of the conserved aspartate residue in this phosphorylation event, Psyr_2449D76A was purified and incubated with autophosphorylated BphP1 (Fig. 7C); this protein was not phosphorylated, demonstrating that the conserved aspartate residue is required for phosphorylation.

Psyr_2449 (SmpR) promoted swarming motility independently of BphP1 and LOV and did not influence lesion formation on pods or leaf colonization

To determine if Psyr_2449 regulates swarming motility, we evaluated the behavior of a *Psyr_2449* deletion mutant. The reduced ability of this mutant to swarm (Fig 8) indicated that it

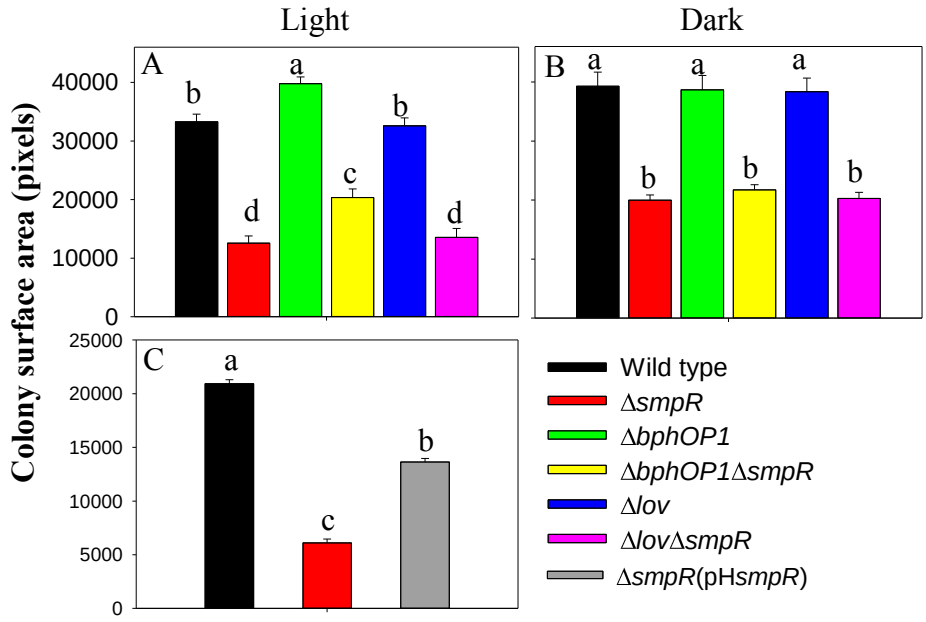

Figure 8. Reduced swarming motility of *smpR* deletion mutants demonstrates that SmpR positively regulates swarming motility. Δ*smpR,* Δ*bphOP1*, Δ*bphOP1*Δ*smpR,* Δ*lov,* Δ*lov*Δ*smpR*, and Δ*smpR*(pH*smpR*) were compared in (A,C) white light or (B) dark conditions. Values and error bars are as described in Fig. 2 ($P<0.05$, two-way ANOVA, n=5).

promotes swarming motility; thus, we designated this gene *smpR* for swarming motility promoting regulator gene. Δ*smpR* was significantly reduced in its ability to swarm in both light (Fig. 8A) and dark (Fig. 8B) conditions, suggesting that it promotes swarming motility independently of light. When *smpR* was expressed under the control of a high-expression promoter on pH*smpR* in Δ*smpR*, swarming was at least partially restored (Fig. 8C), supporting a role for SmpR in promoting swarming motility.

To determine if this regulation involves interactions with BphP1 and LOV, we evaluated the swarming motility of Δ*bphOP1*Δ*smpR* and Δ*lov*Δ*smpR*. Δ*smpR* swarmed significantly less than Δ*bphOP1*Δ*smpR* in the light (Fig. 8A), demonstrating that BphP1-mediated repression still occurs in Δ*smpR*. Moreover, this BphP1-mediated repression did not occur in the dark, based on the similarity in the swarming of Δ*smpR* and Δ*bphOP1*Δ*smpR* (Fig. 8B). Collectively, these results indicate that SmpR regulation occurs via a distinct pathway from BphP1 regulation and that SmpR regulation is not dependent on light. These results do not exclude the possibility that BphP1 may attenuate activation by SmpR; this could potentially involve BphP1 phosphorylation of SmpR, as described above. Such attenuation of SmpR-activated swarming would likely be obscured by SmpR-independent BphP1 repression of swarming in these assays. We previously demonstrated that LOV-mediated regulation of swarming occurs only through its attenuation of BphP1 blue-light sensing (16); therefore, the finding that SmpR regulation occurs via a distinct pathway from BphP1 indicates that SmpR does not act in the same pathway as LOV.

To determine if SmpR acts with BphP1 to regulate lesion formation on bean pods, we inoculated Δ*bphOP1*, Δ*bphOP1(*pN*bphOP1)*, Δ*smpR*, and Δ*smpR*(pH*smpR*) into bean pods, exposed them to white light and measured the sizes of the water-soaked lesions that developed. Δ*smpR* and Δ*smpR*(pH*smpR*) did not differ significantly from the wild type in the sizes of the

lesions that they induced (Fig. 9A), demonstrating that SmpR does not regulate lesion development. We also evaluated Δ*smpR* and Δ*smpR*(pH*smpR*) for their ability to colonize leaves and found them to have comparable fitness to the wild type (Fig. 9B), demonstrating that SmpR does not contribute to leaf colonization.

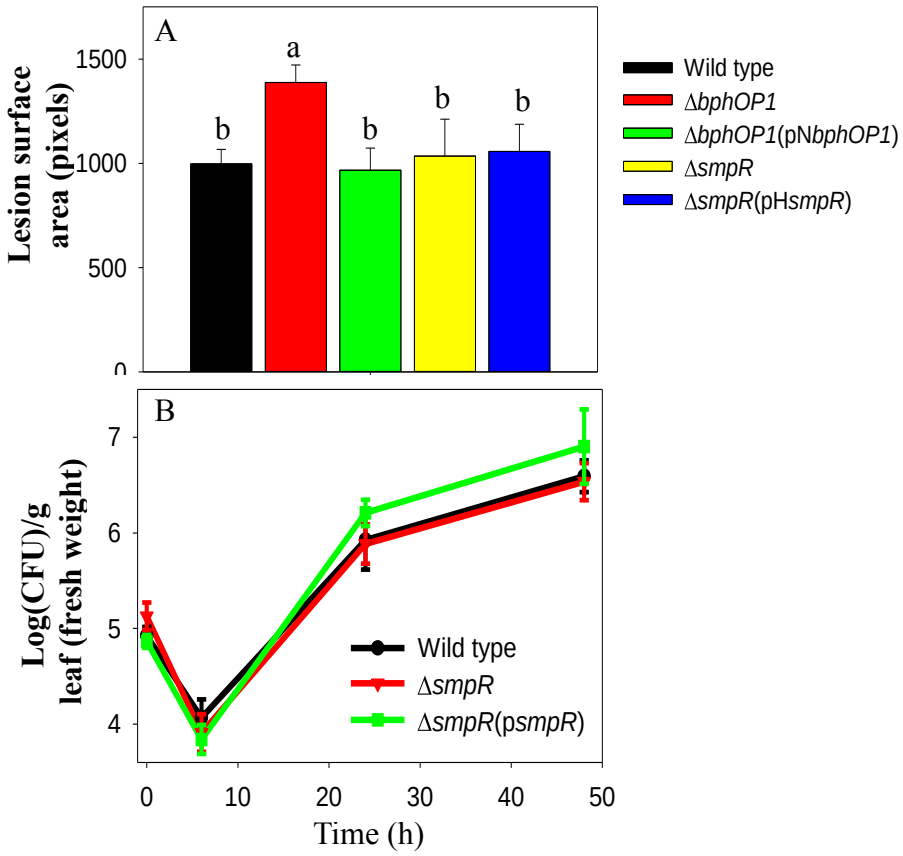

Figure 9. SmpR did not influence lesion development on bean pods or leaf colonization. (A) Lesion sizes of the wild type, Δ*bphOP1*, Δ*bphOP1*(pN*bphOP1),* Δ*smpR*, and Δ*smpR*(pH*smpR*) were compared at 36-48 h after inoculation and incubation in white light. (B) Total populations of wild type, Δ*smpR*, Δ*smpR*(pH*smpR)* were evaluated at 6, 24 and 48 h after inoculation onto leaves. Values and error bars are as described in Fig. 2 ($P<0.05$, one-way ANOVA, n= 9; 12).

Psyr_2699 (Lsr) was encoded by a gene in an operon with *lov* and functioned downstream of BphP1 and LOV in regulating swarming motility

In another approach to identify candidate components of the BphP1/LOV pathway, we focused on a gene downstream of *lov*. The suggestion that *Psyr_2699* is in an operon with *lov* in

P. syringae pv. tomato DC3000 (32) prompted us to explore if *Psyr_2699* and *lov* are co-expressed and to evaluate the role of Psyr_2699 in swarming motility. To determine if B728a *lov* is co-transcribed with the downstream gene, we amplified cDNA from the intra- and intergenic regions of *lov* and *Psyr_2699*. The amplification of fragments using primer sets 2 and 3 with cDNA but not RNA (Fig. 10) indicated that the intergenic region was transcribed. However, the lower intensity bands from the intragenic than intergenic regions indicate possible post-transcriptional modification or secondary structure that reduced the efficiency of PCR amplification.

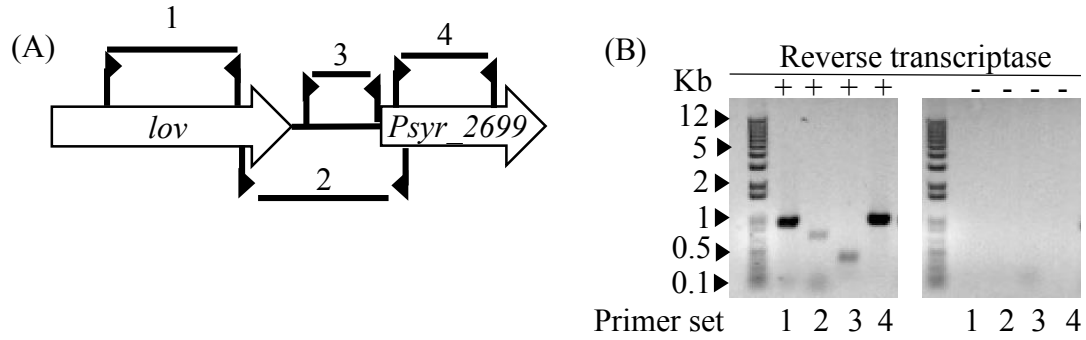

Figure 10. The genes *lov* and *Psyr_2699 (lsr)* were transcribed as a polycistronic mRNA. Total RNA was extracted from cells grown in King's B medium for 5 h to a density of $4x10^8$ cells/ml. PCR amplification using (A) primer sets targeting four regions of the Psyr_2699-2700 (*lov-lsr*) locus showed (B) the presence of amplified bands in all four target regions when reverse transcriptase was present to generate cDNA but not when reverse transcriptase was absent.

To determine if Psyr_2699 was involved in regulating LOV- and BphP1-mediated phenotypes, we examined the behavior of a *Psyr_2699* deletion mutant. The hyperswarming of this mutant (Fig. 11) demonstrated that, like BphP1, it represses swarming motility; thus, we designated this gene *lsr* for LOV-co-transcribed swarming repressor. Δ*lsr* exhibited hyperswarming in the light but not the dark (Fig. 11), much like Δ*bphP1* (16), demonstrating that Lsr repression of swarming motility is light dependent. Δ*lov-lsr* swarmed significantly more than

the wild type much like Δ*lsr* (Fig. 11A), despite the often reduced swarming of Δ*lov*. This demonstrates that loss of *lsr* is phenotypically dominant to loss of *lov* and that Lsr acts downstream of LOV. The decreased swarming phenotype of Δ*lov* seemed to be more sensitive to environmental conditions so the reduction in surface area was variable; however, while the difference between Δ*lov* and the wild type were not significant, Δ*lov* was consistently slightly reduced. Δ*bphOP1*Δ*lsr* resulted in swarming comparable to Δ*bphOP1* and Δ*lsr* (Fig. 11C, D). Loss of both *bphP1* and *lsr* did not result in swarming higher then loss of either alone, suggesting that BphP1 and Lsr are repressing swarming motility through the same pathway. Expression of *lsr* under the control of a high-expression promoter on pH*lsr* in Δ*lsr* and Δ*bphOP1* restored swarming to wild-type levels in the light (Fig. 12A), providing further evidence that Lsr

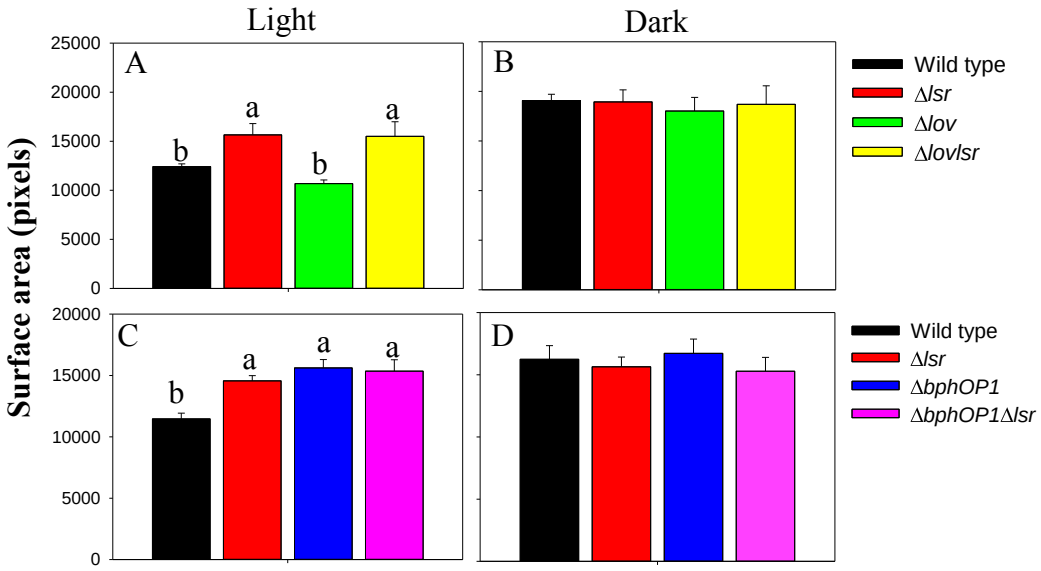

Figure 11. Increased motility of the *lsr* deletion mutants indicated that Lsr functioned with BphP1 to negatively regulate swarming motility. The swarming of the wild type, Δ*lsr,* Δ*lov,* Δ*lov-lsr,* Δ*bphOP1*, and Δ*bphOP1*Δ*lsr* were evaluated in (A,C) white light and (B, D) dark conditions as described in Fig. 8. Values and error bars are as described in Fig. 2. ($P<0.05$, two-way ANOVA, n=5).

represses swarming motility and does so through the same pathway as BphP1. The wild type, Δ*lsr,* and Δ*bphOP1* did not differ significantly in swarming in the dark (Fig. 12B), whereas

Δ*lsr*(pH*lsr*) and Δ*bphOP1*(pH*lsr*) were significantly reduced compared to Δ*lsr* and Δ*bphOP1*. This reduction may have been due to the high level of expression of *lsr*.

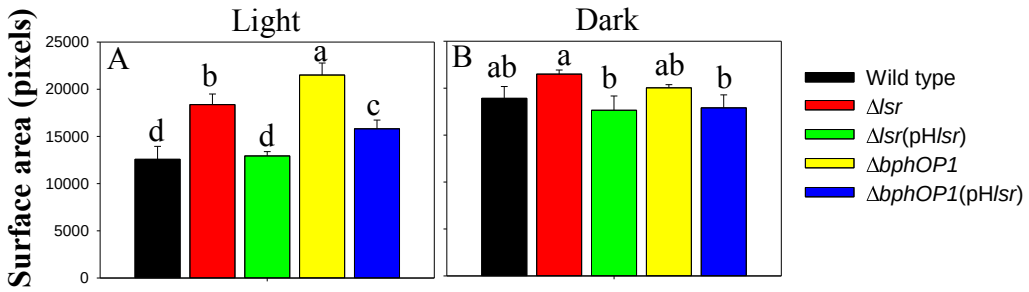

Figure 12. Expression of *lsr* in Δ*lsr* and Δ*bphOP1* indicated that Lsr functioned with BphP1 to negatively regulate swarming motility. The swarming of the wild type, Δ*lsr,* Δ*lsr*(pH*lsr*), Δ*bphOP1*, and Δ*bphOP1*(pH*lsr*) were evaluated in (A) white light and (B) dark conditions as described in Fig. 8. Values and error bars are as described in Fig. 2 ($P<0.05$, two-way ANOVA, n=5).

Lsr functioned downstream of BphP1 in regulating swarming initiation and lesion development on bean pods, but did not contribute to leaf colonization

To determine if Lsr and BphP1 act together to regulate the development of lesions on beans, we evaluated the wild type, Δ*lsr,* Δ*lsr*(pH*lsr*), Δ*bphOP1* and Δ*bphOP1*(pN*bphOP1*) strains in our bean pod virulence assay on beans incubated in the light. Like Δ*bphOP1,* Δ*lsr* formed larger water-soaked lesions than the wild type (Fig. 13A), suggesting that Lsr is also involved in repressing lesion formation. Introducing pH*lsr* intro Δ*lsr* restored lesion formation to levels similar to the wild type, further supporting that Lsr is acting with BphP1 to regulate lesion formation. The wild type, Δ*lsr,* and Δ*lsr*(pH*lsr*) were also compared in their ability to colonize leaves to determine if Lsr contributes to colonization. Both Δ*lsr* and Δ*lsr*(pH*lsr*) established similar population sizes to the wild type at all of the time points evaluated (Fig. 13B), demonstrating that Lsr is not involved in BphP1-mediated contributions to leaf colonization.

To determine if Lsr represses swarming initiation in response to light, we evaluated the timing of tendril formation in Δ*lsr,* Δ*lsr*(pH*lsr*), and the wild type in white, red and blue light. In both white and red light, Δ*lsr* initiated swarming earlier than the wild type (Fig. 14A, B), whereas expression of *lsr* in Δ*lsr* resulted in tendril formation similar to the wild type.

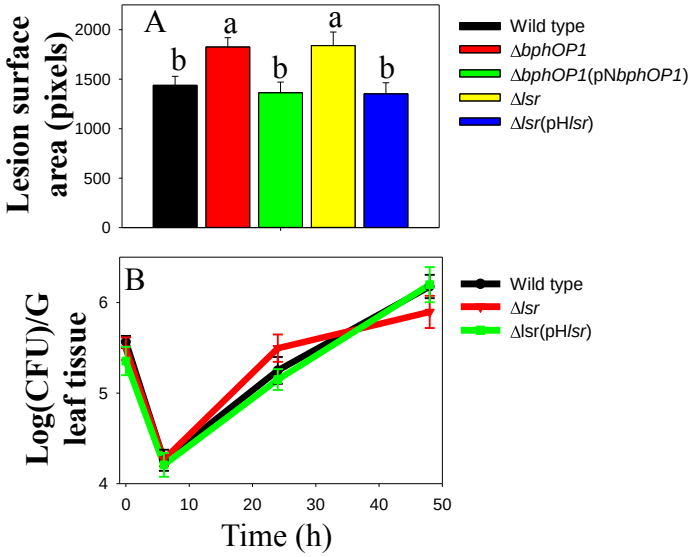

Figure 13. Lsr negatively regulated lesion size through the same pathway as BphP1, but it did not influence leaf colonization. (A) Lesion sizes of the wild type, Δ*bphOP1*, Δ*bphOP1*(pN*bphOP1), Δlsr*, and Δ*lsr*(pH*lsr*) were compared in white light after 36-48 h. (B) Total populations of the wild type, Δ*lsr*, and Δ*lsr*(pH*lsr)* at 6, 24 and 48 h after inoculation onto leaves. Values and error bars are as described in Fig. 2 (A, P<0.05, two-way ANOVA comparing strain and experiment, n=6; B, one-way ANOVA, n=12).

To summarize, these results indicated that Lsr represses swarming initiation in response to red light. Alternatively, under blue light the wild type, Δ*lsr*, and Δ*lsr*(pH*lsr*) did not differ in the timing of swarming initiation (Fig. 14C), suggesting that Lsr does not respond to blue light in its regulation of swarming initiation.

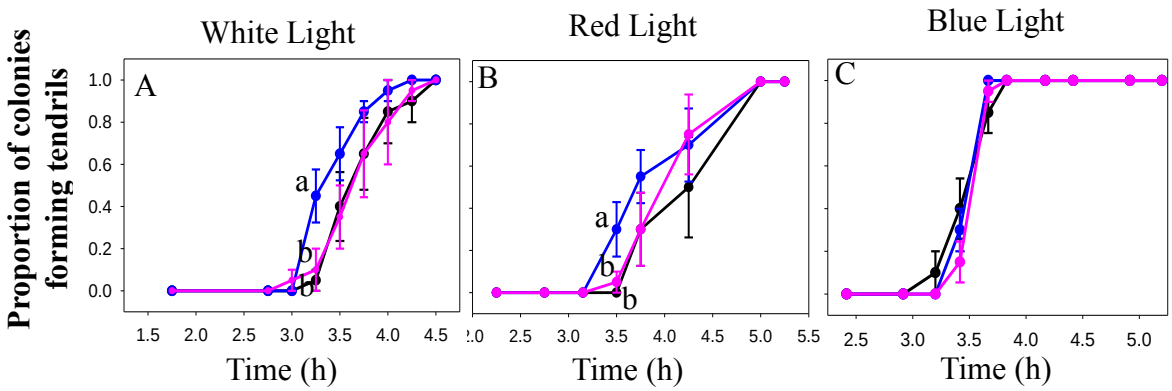

Figure 14. Lsr regulates swarming initiation in response to red but not blue light. The timing of tendril formation was compared in the wild type, Δ*lsr*, and Δ*lsr*(pH*lsr*) in (A) white, (B) red, and (C) blue light. Values and error bars are as described in Fig. 6. (P<0.05, one-way ANOVA on arcsine transformed data, n=4).

A quorum molecule functioned downstream of BphP1 and Lsr in negatively regulating swarming initiation

The N-acyl homoserine lactone (AHL) quorum molecule produced by B728a, 3-oxo-hexanoyl homoserine lactone, is involved in swarming initiation and formation of water-soaked lesions (27). Mutants lacking the AHL synthase AhlI or the regulator AhlR initiated tendril formation earlier than the wild type (Fig. 15), much like a mutant lacking the AHL regulator

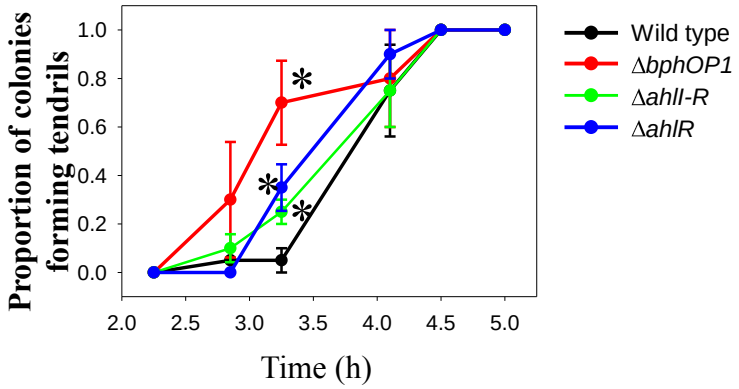

Figure 15. BphP1 has a larger effect than AHL production in regulating swarming initiation. (*) represents values that differ significantly from that of the wild type. (P<0.05, unpaired Student's *t*-test on arcsine normalized values in a comparison with the wild type, n=4).

AefR in a previous study (27). However, these mutants did not initiate swarming as early as Δ*bphOP1* did, suggesting that if AHL regulation occurs in the BphP1 pathway, then it occurs downstream of BphP1.

Amendment of the inoculum with a commercial AHL, N-(β-ketocaproyl)-L-homoserine lactone, did not significantly alter the behavior of the wild type in white or red light, whereas it delayed swarming initiation by Δ*bphOP1* and Δ*lsr* in both white and red light (Fig. 16). The

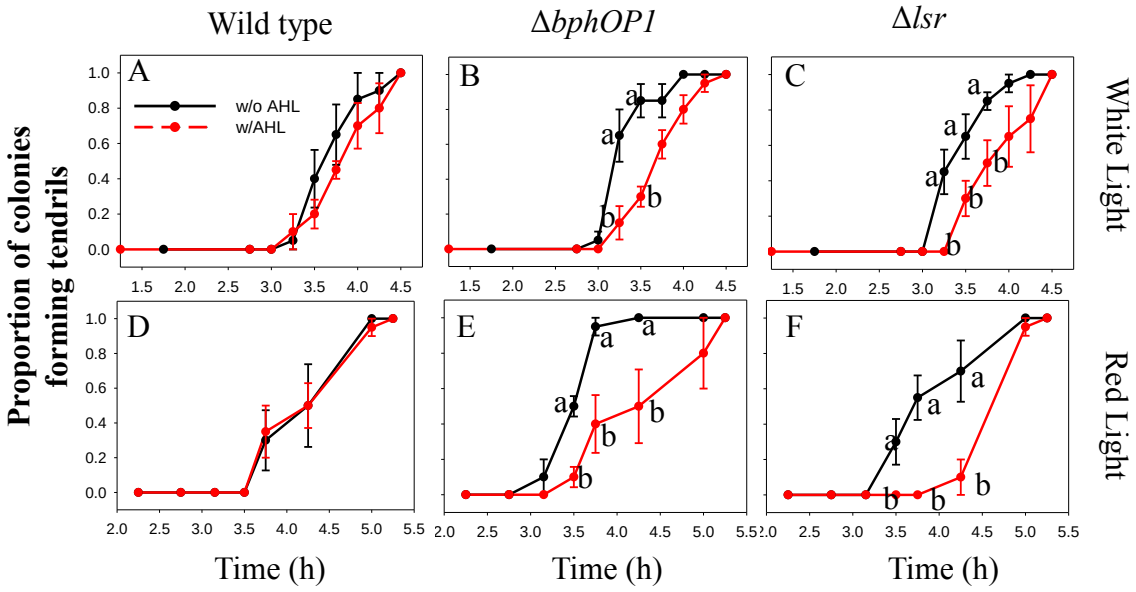

Figure 16. Addition of an AHL alters the behavior of Δ*bphOP1* and Δ*lsr*, but not the wild type, in white and red light. The time until swarming was initiated was examined for the wild type, Δ*bphOP1*, and Δ*lsr* with and without 0.1 µM N-(β-ketocaproyl)-L-homoserine lactone under (A, B, C) white and (D, E, F) red light. Values and error bars are as described in Fig. 2. ($P<0.05$, two-way ANOVA on arcsine normalized data in a single light treatment where strain and presence of AHL were compared, n=4).

impact of the AHL on these mutants indicates that the AHL may function downstream in the BphP1/Lsr pathway to regulate swarming initiation. The lack of an effect of AHL amendment on the wild type may be due to saturation of the wild type with native AHLs, whereas the altered behavior of Δ*bphOP1* and Δ*lsr* may reflect reduced production of native AHLs in these mutants. In summary, the response of Δ*bphOP1* and Δ*lsr* to the addition of the AHL coupled with the

behavior of Δ*ahlI-R* and Δ*ahlR* support a model in which BphP1 and Lsr regulate swarming initiation through the regulation of AHL production.

Discussion

In this work we provide the first evidence for a role of a bacteriophytochrome in plant colonization and virulence. We demonstrated that BphP1 contributes to survival on leaves at the early stages of colonization and may negatively impact colonization at later stages, as well as represses swarming, the development of water-soaked lesions on bean pods, and motility in soil. Moreover, we demonstrated that Lsr, a protein encoded by a gene that is co-transcribed with *lov*, and the quorum molecule AHL are downstream components of the BphP1 regulatory pathway. The BphP1-Lsr-AHL pathway regulates swarming by delaying swarming initiation, suggesting that this pathway represses a switch from a sessile lifestyle to active swarming. We identified SmpR as an additional regulator of swarming motility that interacts with BphP1 *in vitro,* but found that it did not regulate swarming motility via the same pathway as BphP1 and did not influence other BphP1-mediated phenotypes. In sum, this work showed a role for the bacteriophytochrome BphP1 in *P. syringae* interactions with its host and identified at least some of the components involved in this signal transduction pathway.

The contribution of BphP1 to *P. syringae* colonization of leaves is complex. Following inoculation onto leaves *P. syringae* populations decrease, in part due to desiccation stress, and loss of *bphP1* resulted in decreased survival at this stage (Fig. 4). This result demonstrated that BphP1 positively contributes to adaptation to the leaf environment. Differences in survival of Δ*bphP1* and the wild type could be due to hyperswarming of Δ*bphP1* (48) decreasing the formation of aggregates, which are known to enhance survival during desiccation stress (49). The eventual growth of Δ*bphP1* beyond that of the wild type could be due to hyperswarming

enabling greater access to nutrients. However, Lsr and SmpR, which both contribute to regulation of swarming motility, do not contribute to leaf colonization, suggesting that BphP1-mediated contributions to leaf colonization may be independent of swarming motility and that BphP1 regulation of colonization is independent of Lsr.

Red light increases the virulence and population size of *P. syringae* pv. tomato DC3000 but reduces *P. syringae* pv. syringae B278a populations, suggesting that B728a and DC3000 respond differently to red light during leaf colonization. These strains represent two distinct monophyletic groups within the species (3, 50). Distinct responses to red light, which is among the dominant wavelengths in the morning and evening (51), may contribute to the differing abilities of B728a and DC3000 to survive as epiphytes. B728a generally establishes large populations on leaf surfaces, whereas DC3000 establishes only small populations and exhibits poor survival on leaf surfaces (52). Negative regulation of swarming in red light may enable B728a to aggregate at favorable sites immediately following immigration, as suggested by the decreased survival of Δ*bphP1* during early stages of colonization. In contrast, positive regulation of swarming in red light may cause DC3000 to colonize surface sites in which it is subjected to unfavorable environmental conditions, with the cells that reach the apoplast achieving a higher probability of survival (33). Further analysis is needed to test this hypothesis and determine the role of BphP1 in DC3000 red light mediated phenotypes.

BphP1-mediated regulation of swarming motility contributes to *P. syringae* movement in soil. A role of red light sensing in the soil may be conserved among plants and bacteria, as supported by our results and the altered behaviors of plant phytochrome mutants (53). Longer wavelengths of light like red and far-red light are better able to penetrate soil than blue light (54), and shifts in ratios of red light to far-red light are sensed by plant phytochromes to regulate seed

germination (53). We demonstrated that *P. syringae* BphP1 negatively regulated movement from the soil to seeds. The biological significance of light-mediated regulation of movement in the soil is not yet known, but we speculate that periods when significant quantities of red light penetrate soil likely correlate with low water availability as times of high light intensity occur during the warmest and driest times of day, during which bacterial aggregates could protect from desiccation stress.

Lsr represses swarming downstream of BphP1 in response to light through regulation of swarming initiation, which likely contributes to Lsr and BphP1 repression of lesion formation. We demonstrate that mutants lacking *bphP1* or *lsr* hyperswarmed in light and initiated swarming earlier than the wild type when exposed to white and red light, but not blue light. To summarize, these results support a model where BphP1 and Lsr repress swarming motility by delaying swarming initiation in response to red light (Fig. 17). Additionally, like BphP1, Lsr is involved

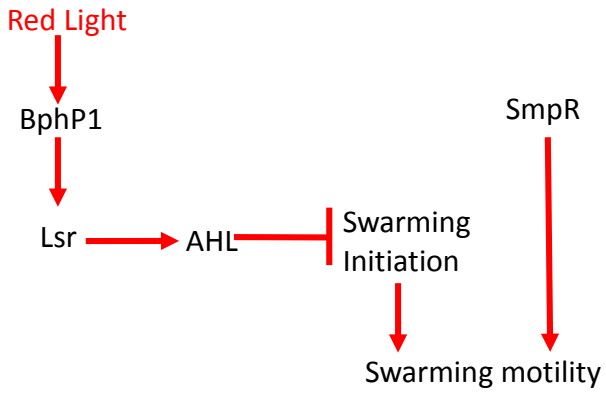

Figure 17. Model of the swarming regulation by BphP1, Lsr, and SmpR. BphP1 and Lsr repress swarming motility in response to light, in part through their regulation of AHL production. However, they may also regulate swarming motility through other mechanisms. SmpR regulates swarming motility through an independent pathway in response to an unknown stimuli.

in repression of lesion formation, as demonstrated by loss of *bphP1* and *lsr* resulting in larger water-soaked lesions than the wild type (Fig. 4)(Fig. 13A). BphP1 and Lsr-mediated regulation

of lesion formation likely occurs due to their involvement in regulating swarming motility. Water-soaked lesion formation by *P. syringae* is hypothesized to be a result of release of water from plant tissue; however, the mechanisms involved in inducing the release of water is not understood. Increased swarming motility may allow the bacteria access to plant tissues farther from the point of inoculation resulting in larger water-soaked lesions. The hyperswarming mutants Δ*ahlIR* and Δ*ahlR* also produced larger water-soaked lesions than the wild type (27), supporting the connection between lesion formation and swarming motility. Tissue maceration also occurs in late stages of water-soaked lesion formation and is predicted to be the result *of P. syringae* enzymes that disrupt plant cells, which is the mechanism of lesion formation by *Erwinia* spp. (55). In addition to increased lesion development, Δ*ahlIR* and Δ*ahlR* showed reduced tissue maceration, suggesting a role of AHL in regulating tissue maceration virulence determinants (27), which may be modulated by BphP1.

BphP1 and Lsr regulate swarming initiation, at least in part, by activating AHL synthesis. Δ*ahlIR* and Δ*ahlR,* which lack the ability that responds to AHL, both initiated swarming earlier than the wild type but not as early as Δ*bphP1* (Fig. 15), demonstrating that loss of *bphP1* has a larger effect on swarming initiation than loss of AHL production. This result is consistent with previous studies where loss of AHL production resulted in hyperswarming and swarming initiation in both *P. syringae* and *P. aeruginosa* (27, 56) and also suggests that BphP1 acts upstream of AHL synthesis in regulating swarming initiation. BphP1-mediated AHL activation is supported by the altered timing of tendril formation of Δ*bphOP1* and Δ*lsr* when AHL was added to swarm colonies exposed to red and white light (Fig. 16). The lack of an effect of AHL amendment on the behavior of the wild type is likely because it is already showing a maximal response to AHL due to AHL production through BphP1 and Lsr and the fact that AHL

production is autoregulated (57). BphP1 and Lsr mediated regulation of swarming motility is likely not due to their regulation of swarming initiation exclusively, as repression of swarming initiation by addition of AHL did not completely abolish hyperswarming in mutants lacking *lsr* and *bphP1* (data not shown).

AHL regulation by BphP1 is consistent with our model for BphP1-mediated regulation of leaf colonization, as loss of *bphP1* and AHL production would result in a decreased ability to coordinate density-dependent responses in aggregates (58). Additionally, mutants lacking the ability to produce AHLs were decreased in their ability to survive desiccation stress on leaves (27) much like the reduced survivability of Δ*bphOP1* in early stages of colonization. Alginate which protects from desiccation stress (59) and reactive oxygen intermediates (60), is regulated by AHL production (43) and likely has a role in the increased tolerance to environmental stress exhibited by aggregates. A role for alginate in BphP1-mediated regulation of leaf colonization has not yet been investigated; however, we evaluated BphP1 for a role in sensitivity to the ROI hydrogen peroxide and found that mutants lacking *bphP1* did not differ in sensitivity compared to the wild type (data not shown). Taken together our results support a model in which BphP1 and Lsr act together to promote AHL production and repress swarming initiation (Fig. 17).

SmpR interacts with BphP1 *in vitro* and regulates swarming motility; however, it does not act with BphP1 to regulate any known BphP1-mediated phenotypes. A bioinformatics search and a screen of BphP1-mediated phosphorylation identified SmpR as a potential component of the BphP1-mediated signal transduction pathway (Fig. 6). Whereas loss of *smpR* reduced swarming motility, the swarm colony surface area of Δ*smpR*Δ*bphOP1* in the light was intermediate between those of Δ*smpR* and Δ*bphOP1* (Fig. 8), demonstrating that BphP1 can repress swarming in Δ*smpR* and that BphP1 and SmpR regulate swarming through two

independent pathways. SmpR was also not involved in BphP1-mediated regulation of leaf colonization or water-soaked lesion formation; however, it could be involved in unidentified BphP1-regulated phenotypes based on the behavior of other photoreceptors such as those in *Synechocystis* sp. strain PCC 6803 (61), *X. axonopodis* pv. citri (62), *Caulobacter crescentus* (63, 64), and *R. leguminosarum* (65). Evidence for a role of BphP1 in pathways affecting colonization that are independent of motility include the lack of altered colonization phenotypes in Δ*smpR* and Δ*lsr*, which are both altered in motility. The *in vitro* interaction of SmpR and BphP1 could also be explained by non-specific cross-talk. If the cognate response regulator for an interacting histidine kinase is not present, the histidine kinase is more likely to phosphorylate an alternative target (38).

Collectively, this work further elucidates the physiological role of BphP1 in *P. syringae* and provides the first evidence of a bacteriophytochrome regulating *P. syringae* phenotypes that affect plant colonization. In addition, we identify two novel proteins involved in swarming motility. Future work will focus on understanding how BphP1 and Lsr interact and identifying additional BphP1-mediated phenotypes. While Lsr acts downstream of BphP1 in regulating swarming motility, it is not acting as the cognate response regulator as it does not have a response regulator domain that could serve as a site for phosphorylation. BphP1 interactions with Lsr could occur through direct protein-protein interactions or through the cognate response regulator of BphP1. This complex interaction could be better understood by screening the *P. syringae* proteome for BphP1- and Lsr-interacting proteins and employing the assays developed in this study to analyze the impacts of these proteins on swarming initiation, swarming motility, water-soaked lesion formation, and colonization of seeds and leaves. Identification of additional

components of BphP1 mediated signal transduction will allow for elucidation of the mechanism of BphP1 contributions to motility and plant colonization and virulence.

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CHAPTER 4. *PSEUDOMONAS SYRINGAE* PV. SYRINGAE B728A TYPE IV PILI, ALGU SIGMA FACTOR, AND ALGINATE BIOSYNTHSIS PROTEIN ALGD CONTRIBUTE TO SWARMING MOTILITY

Abstract

Swarming motility by *Pseudomonas syringae* pv. syringae B728a is known to be regulated by quorum sensing and GacA, in addition to requiring the biosurfactant syringafactin; however, the relative contributions of biosurfactants, the global regulators AlgU and GacA, flagella, and type IV pili to swarming motility and their response to light have not been evaluated. Here we demonstrate that the type IV pilus is a major contributor to swarming motility based on the significant reduction in swarm colony surface area when *pilA*, the prepilin subunit that forms pilin, was deleted. Additionally, we characterize an unusual pattern of surface movement in which a mutant lacking *fliC*, which encodes flagellin, formed hollow arcs around the colony. The formation of these hollow arcs was dependent on functional type IV pili as deletion of both *fliC* and *pilT* resulted in loss of the phenotype. These structures did not form in colonies incubated in the light, suggesting that type IV pili are light regulated. We also show that wild-type cells repel specific mutants, causing movement away from the wild type, and this repellency is lost upon deletion of *rhlA*, which is involved in the synthesis of the biosurfactant 3-(3-hydroxyalkanoyloxy) alkanoic acid. Surprisingly, the wild type repels mutants lacking *gacA* and *syfA*, which do not swarm but exhibit movement, designated here as translocation, in response to the wild type. Production of the polysaccharide alginate promotes swarming motility based on the reduced swarming of an *algD* mutant, suggestive of a role for alginate-mediated hydration in swarming. In contrast, osmotic stress negatively impacts swarming motility. This negative regulation may occur through the osmotically activated sigma factor AlgU based on the

dramatically increased swarming motility of an *algU* mutant. Taken together, this work elucidates the role of type IV pili in swarming motility and identifies two novel patterns of surface motility and factors regulating swarming in *P. syringae*.

Introduction

Movement is a significant component in the virulence of many plant pathogenic bacteria as it promotes their ability to reach preferred habitats for colonization. Many bacterial pathogens require motility for the initial phases of infection, whereas others require it throughout infection or for survival in environmental reservoirs (1). Bacteria utilize a variety of methods to move including swimming, swarming, twitching, gliding and sliding; each type of motility involves a distinct mechanism for movement and occurs under a particular set of conditions (2). Swimming and swarming motility depend on the flagella, twitching requires the type IV pili, sliding is characterized by passive translocation, and several distinct mechanisms of gliding are known (2). Swarming, twitching, gliding, and sliding are all surface-associated movements, whereas swimming occurs in habitats that have sufficient water to enable full flagellar rotation.

Motility is an important mechanism of colonization utilized by the foliar pathogen *Pseudomonas syringae*, which is commonly studied due to its ability to cause disease on economically important crops (3, 4) and as a model for plant-bacterial interactions (5). Mutants lacking motility are reduced in survival on leaves after exposure to ultraviolet radiation and desiccation stress and produce significantly fewer lesions when compared to motile strains, in part due to their inability to move to protected sites (6, 7). Additionally, some hypermotile mutants have increased virulence and survival on plants (8).

The flagellum is a membrane anchored filament that rotates like a propeller to generate force (9). The flagellar motor consists of stators and rotors surrounding the basal body in the

membrane (10, 11). The rotation of the motor is controlled by the switch, which provides directional control of filament rotation in response to changing environmental signals (12). The flagellar hook is exterior to the cell and provides the foundation for the filament, a long, thin helical structure that functions as the propeller (13). Flagellar biosynthesis is an energy-intensive process and is tightly regulated in a hierarchical fashion to ensure efficient and regulated production (14). Flagellar regulation has been well studied in *Pseudomonas aeruginosa*, where the master regulator FleQ controls a four-tiered cascade of production of flagellar components (14). FleQ initiates expression of the Class II genes, which determine the placement of the flagellum and form the basal body and export apparatus (14). FleQ activity is then repressed to maintain monoflagellated status (14); FleQ repression results in activation of Class III genes for completion of the hook-basal body (14). Upon completion of the basal body, expression of *fliC* and *fleL* occurs; these encode the structural protein flagellin and a flagellin length regulator, respectively (14). In *P. syringae* flagellar biosynthesis is coordinated with production of the biosurfactant 3-(3-hydroxyalkanoyloxy) alkanoic acid (HAA), which contributes to tendril formation during swarming motility (15).

Flagella mediate swimming motility when they are in an environment enabling full rotational movement, whereas they are one of a few factors contributing to swarming motility in *P. syringae* on moist surfaces. Swarming motility is characterized as flagella-mediated, coordinated movement across a semi-solid surface that involves a biosurfactant. Syringafactin is a key biosurfactant for swarming motility in the organism used in this study, *P. syringae* pv. syringae strain B728a (16, 17). In some organisms flagella are critical for swarming; for example, loss of *fliC* completely abolished swimming and swarming motility in *P. syringae* pv. tabaci (18, 19). In contrast, *P. aeruginosa* strains lacking *fliC* retained some surface motility

(20), suggesting that swarming may rely on other surface appendages. B728a genes associated with flagellar synthesis and syringafactin synthesis were induced on leaf surfaces, with the flagellar synthesis genes induced at least 4.5-fold more in cells on leaf surfaces than in apoplastic sites (21). Additionally, *P. syringae* pv. tabaci that were unable to swarm exhibited reduced virulence and survival on plants (18, 19). Collectively, these results suggest that swarming motility is an important adaptation during the epiphytic stage of B728a's lifecycle, where it establishes large populations before causing disease (22).

The *P. syringae* transcriptome is regulated by a number of global regulators, a few of which contribute to expression of genes that are associated with swarming motility; however, the impact of this regulation on swarming is not well understood. RpoN, AlgU and GacA are three major regulators that influence the expression of a large number of genes during B728a leaf colonization (21). RpoN regulates expression of flagellar synthesis genes during epiphytic colonization (23). AlgU controls *P. syringae's* response to low water availability, which is a significant environmental stress encountered on the leaf surface (23). AlgU-regulated responses include the synthesis of alginate, which contributes to epiphytic fitness (24-26), and, as a hygroscopic polysaccharide, may contribute to hydrating swarm colonies (27). The two-component system GacA/GacS is also required for swarming motility but does not have a role in swimming motility, although its role in swarming is not understood (28). Taken together these results suggest that the global regulators RpoN, AlgU, and GacA regulate swarming motility and that alginate may contribute to swarming motility.

The type IV pilus mediates twitching motility and has also been shown to contribute to attachment and swarming motility in some bacterial species (20, 29, 30). Twitching motility is the result of repeated pili extension, attachment, and retraction propelling cells across a surface

(31). Twitching requires moderate viscosity and can be observed by individual cells or cells grouped together in rafts along their long axes (32). At least 40 genes located throughout the chromosome are involved in pilus synthesis and assembly (20). PilA is a small prepilin subunit that is cleaved by PilD and then is assembled at the base of the minor pilins by the cytosolic membrane protein PilC (33). Following assembly the pilus protrudes outside of the cell through the PilQ outermembrane pore and is stabilized at its base by PilP (33). After the terminal end of the pilus attaches to a surface, PilT facilitates retraction, with the aid of PilU, through disassembly of the pilus into pilin monomers (33). In *P. aeruginosa* pilus biosynthesis is tightly regulated by the sigma factors RpoN and AlgU in addition to the FimS/AlgR two-component system, supporting a role for AlgU in motility (34-36).

The role of the type IV pilus in swarming motility, colonization, and virulence varies among *P. syringae* strains. In *P. syringae* pv. tomato DC3000, loss of *pilA* resulted in poor attachment to leaves, reduced ultraviolet tolerance, and reduced population sizes, but did not affect virulence based on unaltered disease symptoms (29, 30). In *P. syringae* pv. tabaci 6605, loss of *pilA*, *pilO*, and the minor pilins resulted in impaired swimming motility, complete loss of swarming motility, and reduced virulence and biofilm formation, but did not affect twitching motility (37, 38). These results demonstrate that although flagella have a prominent role in swimming and swarming, pili can contribute to both in *P. syringae* (37, 38), as supported by the finding that loss of *pilA* abolished swarming motility in *P. aeruginosa* (20).

To date, swarming motility in B728a is known to require the biosurfactant syringafactin (17) and to be regulated by quorum sensing (8) and GacA/GacS (28). In this work, we investigated the relative contribution of flagella, type IV pili, the biosurfactants syringafactin and HAA, alginate, and the global regulators AlgU and GacA to the swarming motility of B728a,

including how these contributions are influenced by light. We provide the first documentation for a role of type IV pili in B278a swarming motility and evidence that these pili are regulated by light. We also demonstrate an unusual type IV pili-mediated surface movement pattern in the absence of flagella, and an unusual interaction between colonies that manifests as a movement away from HAA-producing colonies by colonies deficient in swarming motility (16, 28). Lastly, we demonstrate that the sigma factor AlgU strongly represses swarming motility, despite AlgU activation of alginate production (21) and a positive role for alginate in promoting swarming motility. Collectively, this work highlights the complex factors impacting the extent, patterns and regulation of swarming motility in B728a.

Materials and methods

Growth conditions and construction of mutants

The bacterial strains used in this study are described in Table 1. *P. syringae* strains were grown at 25°C in King's B medium (39) with shaking unless otherwise described. Rifampin (Rif) and kanamycin (Km) were added to a final concentration of 50 µg/ml when necessary.

Table 1. Strains and Plasmids used in this study

Strain or plasmid	Description of relevant genotype	Reference or source
P. syringae strain		
B728a	Wild type; Rif^r	(40)
Δ*fliC*	B728a Δ*Psyr_3466*; Rif^r	This study
Δ*pilA*	B728a Δ*Psyr_0799*; Rif^r	This study
Δ*pilT*	B728a Δ*Psyr_0478*; Rif^r	This study
Δ*pilT*Δ*fliC*	B728a Δ*Psyr_0478*Δ*Psyr_3466*; Rif^r	This study
Δ*rhlA*	B728a Δ*Psyr_3129*; Rif^r	This study
Δ*syfA*	B728a Δ*Psyr_2576*; Rif^r	This study
Δ*syfA*Δ*rhlA*	B728a Δ*Psyr_2576*Δ*Psyr_3129*; Rif^r	This study
gacA::Tn5	B728a *Psyr_2897::Tn5*; Rif^r Km^r	(41)

Table 1 continued

Δ*algU*	B728a Δ*Psyr_3958*; Rif^r	(21)
algD::TnlacZ	B728a *Psyr_1063::TnlacZ*; Rif^r Km^r	(42)

Deletion mutants were constructed using splice-overlap-extension PCR deletion mutagenesis as described in Chapter 2. Δ*pilT* and Δ*syfA* were utilized to construct mutants Δ*pilT*Δ*fliC* and Δ*syfA*Δ*rhlA* as described in Chapter 2. Primers for construction and confirmation of each deletion mutant can be found in Table 2.

Table 2. Primers used for this study

Primer[a]	Sequences[b]
Primers for constructing deletion mutants	
fliC-FL1-F	5'-GTACCACTCGGAATAGCACTTGAC-3'
fliC-FL1-R	5'-AGCCTACACAATCGCTCAAGACGTTCGATCCTGGCACAGGCTAACCAG-3'
fliC-FL2-F	5'-AATATCCGGGTAGGCGCAATCACTTGGACGCTCAACGATGTTACGTTG-3'
fliC-FL2-R	5'-CGACCAACGTCGCTGCATTCGATA-3'
pilA-FL1-F	5'-AGCAATACGGTTTGCCAAATGGAT-3'
pilA-FL1-R	5'-AGCCTACACAATCGCTCAAGACGTCTTCTGTGCATTCATATCTACTTC-3'
pilA-FL2-F	5'-AATATCCGGGTAGGCGCAATCACTACCGATTCTGTGTACGCTTTAATTT-3'
pilA-FL2-R	5'-GGTGATTTCAGAACGTTGCTG-3'
pilT-FL1-F	5'-GACTTCAAGGCCTGACAGACGGTT-3'
pilT-FL1-R	5'-AGCCTACACAATCGCTCAAGACGTCTCGGTAATATCCATACAGCTCCT-3'
pilT-FL2-F	5'-AATATCCGGGTAGGCGCAATCACTGGATAACTTCTGATCACAGGGATG-3'
pilT-FL2-R	5'-CTATTCCGACGTATGGCACATGAG-3'
rhlA-FL1-F	5'-CCTGATACTCGACCTGCATGAAC-3'
rhlA-FL1-R	5'-AGCCTACACAATCGCTCAAGACGTCGTTGACCAGCAGAATGGTCTTG-3'
rhlA-FL2-F	5'-AATATCCGGGTAGGCGCAATCACTCACTGAAGCTACCCAGGAGGT-3'
rhlA-FL2-R	5'-GCACTCGCAACGTGCCATTTGCA-3'
syfA-FL1-F	5'-TCCACCGAACTGTTCTTGACCTTG-3'
syfA-FL1-R	5'-AGCCTACACAATCGCTCAAGACGTACTGATCTGATCAAGCCAGACGTC-3'
syfA-FL2-F	5'-AATATCCGGGTAGGCGCAATCACTACGCAGCCATCACAGAGAACATG-3'
syfA-FL2-R	5'-GTGATGCTGTGCTCTTCGATAGCA-3'

[a] FL indicates flanking region

[b] underlined portions of the primer sequence indicate the region that binds to the kanamycin cassette

Assay for quantifying swarming motility

Swarming motility was evaluated as previously described (43) with the altered drying conditions and light exposure conditions described in Chapter 3. Briefly, *P. syringae* strains were grown overnight to late-log phase and were harvested, washed, and normalized with nanopure water to a concentration of $4x10^8$ cells/ml by measuring the optical density (600 nm). Square plates (100 x 100 x 15 mm) containing 30 ml of King's B medium with 0.4% agar were dried in a laminar flow hood for 90 min. Following drying, plates were inoculated with at least five replicate 2-µl drops containing $8x10^5$ cells/ml for each strain, with the drops arranged such that each strain was represented in every row and column. The plates were sealed in parafilm and incubated under fluorescent bulbs (Ecolux F40C50-Eco, General Electric Co., Fairfield, CT) or in the dark by enclosure in two layers of aluminum foil. The swarm colony surface area was quantified using pixel counts in Adobe Photoshop, as previously described (43). Analysis of variance (ANOVA) was performed on pixel counts for each colony for replicate plates incubated in either light or dark conditions, with $n \geq 5$ for each strain, to determine the statistical significance of differences in swarm colony surface area among strains. Light and dark treatments were incubated side-by-side and experiments were terminated at the same time to allow for comparisons across treatments. Swarm plates were incubated at 22-23^{0}C for 10-14 h to quantify swarming, and for 14-17 h to observe the repelling ability of the wild type. Each comparison was performed a minimum of three times.

Results

Type IV pili contribute to tendril formation during swarming motility and influence behavior in a light-dependent manner

To evaluate the relative contribution of flagella and pili in swarming motility, Δ*fliC*, Δ*pilA*, Δ*pilT*, and Δ*pilT*Δ*fliC* were constructed and quantitatively analyzed for swarm colony surface area. Loss of the gene for the structural pilin, *pilA*, resulted in a significant decrease in swarming motility (Fig. 1), suggesting that type IV pili contribute to maximal swarming motility. This decreased swarm colony surface area was associated with a decrease in the width and length of tendrils emanating from the Δ*pilA* colony (Fig. 1B, C), suggesting that type IV pili contribute to maximal tendril formation, and specifically that type IV pili may mediate the cell-to-cell interactions contributing to the structured arrangement of cells into tendrils. This is supported by the continued production of tendrils by the Δ*fliC* mutant in the light and dark (Fig. 1B, C); however, tendril formation by Δ*fliC* was phenotypically distinct in the light versus the dark (Fig. 1B, C). In the light, the tendrils radiated outward as in the wild type, but in the dark they formed hollow arcs around the colony, suggestive of increased cell-to-cell interactions and enhanced type IV pili production in the dark. This Δ*fliC* phenotype demonstrates light regulation of swarm colony morphology, and is consistent with light-mediated reduction of type IV pili production, which is further supported by the lack of light-mediated repression of swarming exhibited by Δ*pilA* (Fig. 1A). Loss of *pilT* results in an inability to retract pili (44), and this resulted in a complete loss of swarming (Fig. 1); this mutation was phenotypically dominant over Δ*fliC*.

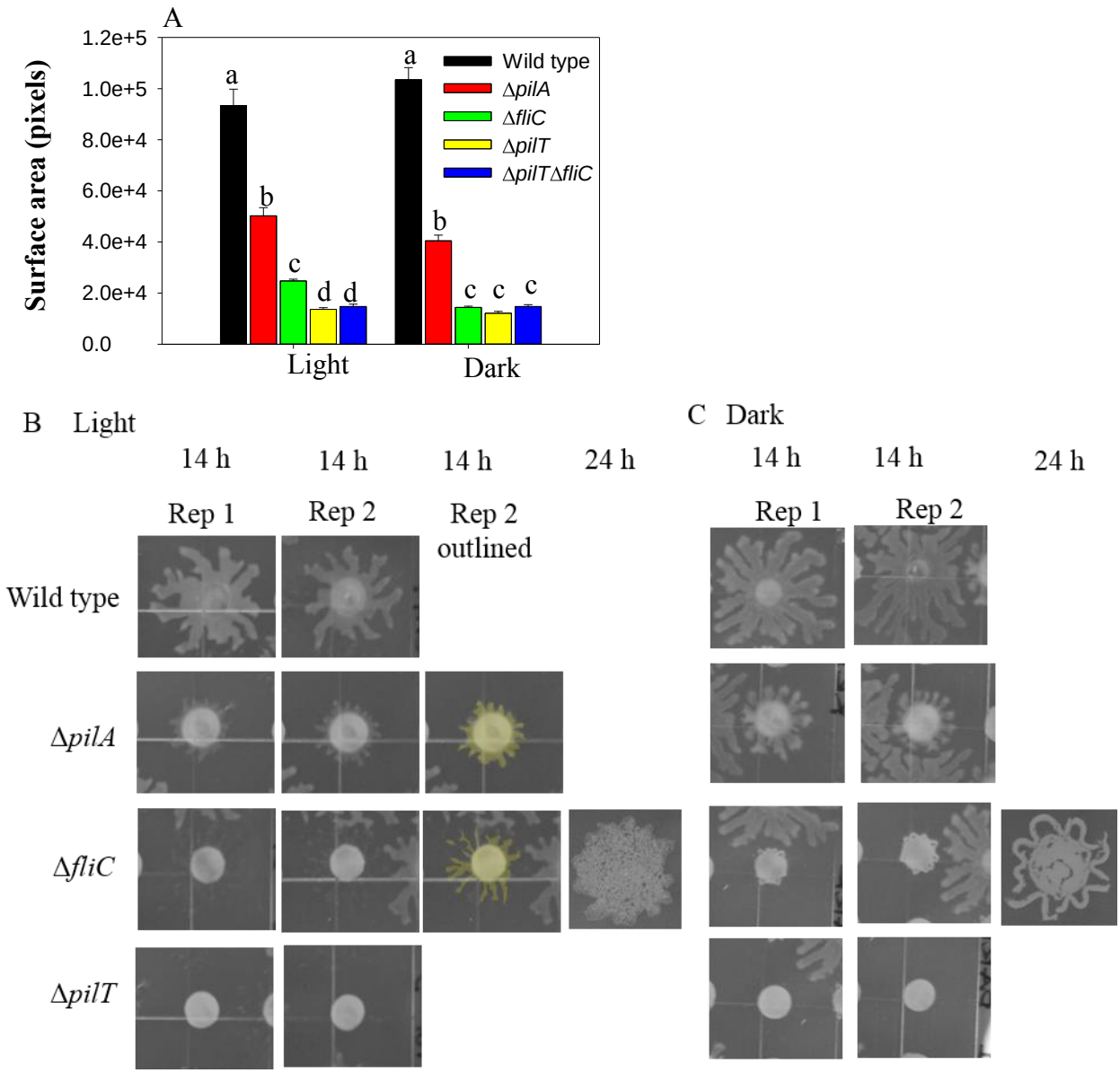

Figure 1. Type IV pili contribute to swarming motility and are light regulated. Swarming motility of the wild type, Δ*pilA,* Δ*fliC,* Δ*pilT,* and Δ*pilT*Δ*fliC* were (A) quantified and (B,C) examined in (A,B) light and (A,C) dark conditions. Photographs were taken at 14 and 24 h to demonstrate phenotypic differences in swarming patterns; tendrils that were particularly transparent were outlined for better visualization. Values in the light conditions or dark conditions that are indicated by the same letter do not differ significantly ($P<0.05$, one-way ANOVA comparing within a treatment, n=5). Error bars represent standard error.

The global regulators AlgU and GacA have opposing roles in regulating swarming motility

AlgU, a sigma factor that regulates the physiological response of cells to water-limiting conditions, is important for B728a cells on leaf surfaces (21). To explore the impact of AlgU and water limitation on swarming motility, we first evaluated if increased osmotic stress influenced swarming motility. When the wild type was inoculated on plates containing 0.05, 0.1, or 0.15 M NaCl, the swarm colony surface area decreased with increasing NaCl concentration, although the difference between 0.1 M and 0.15 M NaCl was not significant (Fig. 2A). These decreases were

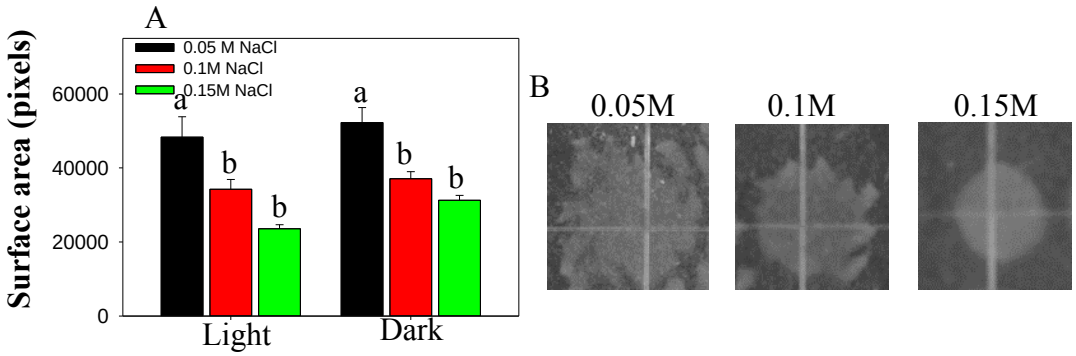

Figure 2. Osmotic stress reduced the swarming motility of B728a, and this reduction was not altered by light. (A) The swarm colony surface area was reduced, and (B) and the pattern of tendril formation was altered, in the presence of increasing concentrations of sodium chloride. Light did not influence this behavior. Values and error bars are as described in Fig. 1 ($P<0.05$, one-way ANOVA, n=5).

accompanied by a loss of tendril formation, resulting in swarm colonies with a smooth circular edge at 0.15 M NaCl (Fig. 2B). This osmotic repression of swarming motility was not influenced by exposure to light (Fig. 2A).

To determine if AlgU is involved in osmotic stress-mediated repression of swarming, the swarming motility of Δ*algU* was evaluated. Δ*algU* swarm colonies were dramatically larger than those of the wild type (Fig. 3), demonstrating that AlgU acts as a repressor of swarming motility. These results are consistent with the possibility that AlgU contributes to the osmotic stress-mediated repression of swarming motility in the wild type (Fig. 2). Δ*algU* exhibited less

swarming motility in the light than the dark, which is consistent with the behavior of the wild type (43), demonstrating that AlgU is not involved in light-mediated regulation of swarming motility.

Alginate is a hygroscopic polymer that creates a hydrated microenvironment as an adaptation when water availability is low (45, 46); the genes involved in alginate biosynthesis are highly expressed on leaves (21) and significantly contribute to virulence and epiphytic fitness (25, 26). Hygroscopic polymers like alginate have been suggested to contribute to hydration of swarm colonies by causing water to flow from the medium into the swarm colony, thus promoting motility (27). AlgD catalyzes the first step in the alginate biosynthetic pathway (46). *algD::TnlacZ* was evaluated to determine if alginate contributes to swarming motility in *P. syringae*. Loss of *algD* resulted in slightly decreased swarming in the light and significantly decreased swarming in the dark (Fig. 3), suggesting that alginate positively contributes to swarming motility. GacA is a global regulator in *P. syringae* both in culture (47) and on leaves (21). *gacA::Tn5* was completely non-motile (Fig. 3), as observed previously (28).

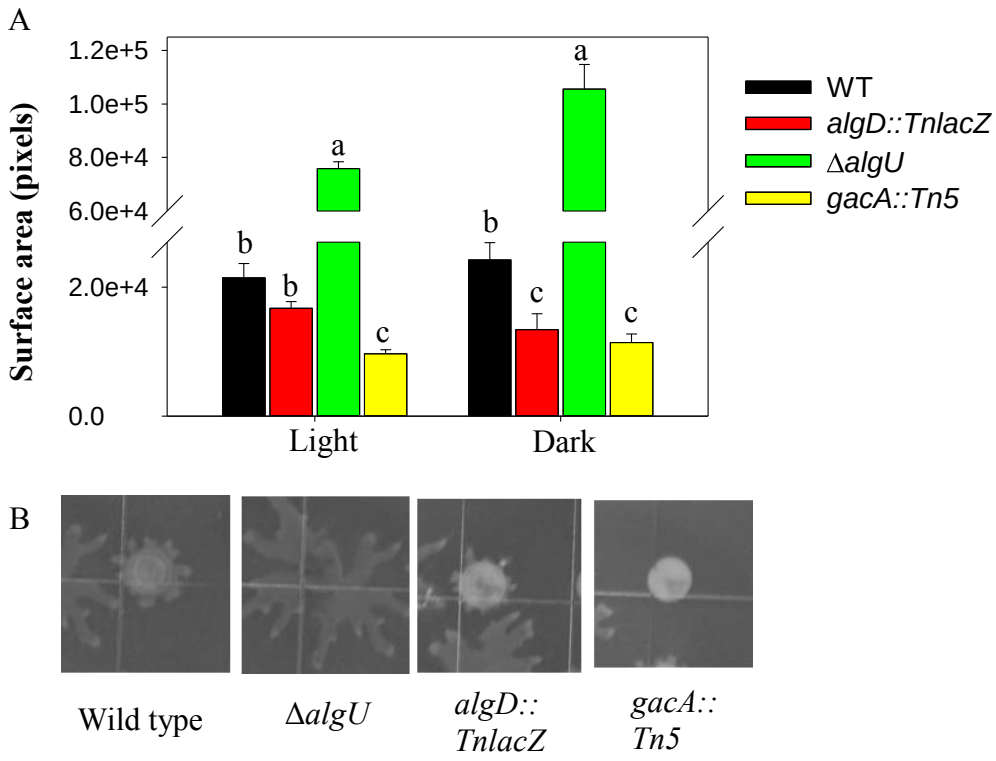

Figure 3. AlgU negatively regulates swarming motility, whereas AlgD and GacA promote it; however, light does not influence this regulation. Swarming motility of the wild type, *algD::TnlacZ,* Δ*algU,* and *gacA::Tn5* were (A) compared in light and dark conditions and (B) photographed. Results were analyzed as described in Fig. 2. Values and error bars are as described in Fig. 2 ($P<0.05$, one-way ANOVA comparing within a treatment, n=6).

SyfA is critical for swarming motility, while RhlA functions in the development of tendril formation

Biosurfactant production is a critical component of swarming motility (15, 17). To determine the role of biosurfactant production in light-mediated regulation of swarming motility, the surface area of Δ*syfA,* Δ*rhlA,* and Δ*syfA*Δ*rhlA* swarm colonies was quantitatively analyzed in light and dark conditions. Loss of *syfA* resulted in complete loss of swarming motility (Fig. 4);

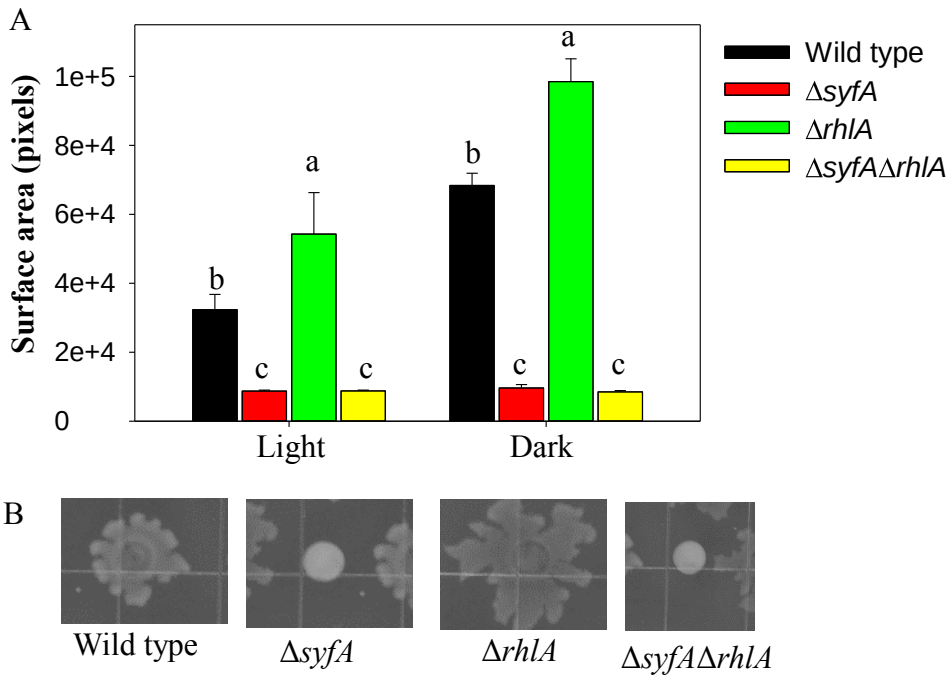

Figure 4. Loss of *syfA* completely abolishes swarming motility, whereas loss of *rhlA* results in increased swarm colony surface area. Swarming motility of the wild type, Δ*syfA,* Δ*rhlA,* and Δ*syfA*Δ*rhlA* were compared in (A) light and dark conditions and were (B) photographed. Results were analyzed as described in Fig. 2. Values and error bars are as described in Fig. 2 ($P<0.05$, one-way ANOVA comparing within a treatment, n=6).

this result was in contrast to analysis by Burch et. al 2010 (17), in which loss of *syfA* significantly reduced swarming but did not completely abolish it. This result supports the role of syringafactin as the primary surfactant utilized during swarming motility. In contrast to Δ*syfA,* Δ*rhlA* produced swarm colonies that had a significantly larger surface area and often produced fewer tendrils than the wild type (Fig. 4). This result is consistent with the prediction of Burch et al 2012 (15) that HAA acts as a repellent that ensures separation between adjacent tendrils. Δ*rhlA* was reduced in swarming motility in the light compared to the dark, demonstrating that light-mediated repression of swarming motility occurs independently of HAA. Δ*syfA*Δ*rhlA* also exhibited a complete lack of swarming motility (Fig. 4), demonstrating that loss of *syfA* is phenotypically dominant to loss of *rhlA*.

Strains lacking *syfA* or *gacA*, but not *pilT*, *algU*, *algD*, *fliC* or *pilA*, exhibit rapid movement away from the wild type, and this repellency is due, at least in part, to HAA production

Loss of *syfA* and *gacA* resulted in cells that no longer swarm on semi-solid agar plates (Fig. 3, 4); however, we observed that Δ*syfA* and *gacA::Tn5* swarm colonies actively moved away from wild-type swarm colonies that were in close proximity (Fig. 5). When the wild-type tendrils reached within 5 mm of a Δ*syfA* or *gacA::Tn5* swarm colony (see arrows in Fig. 5), the

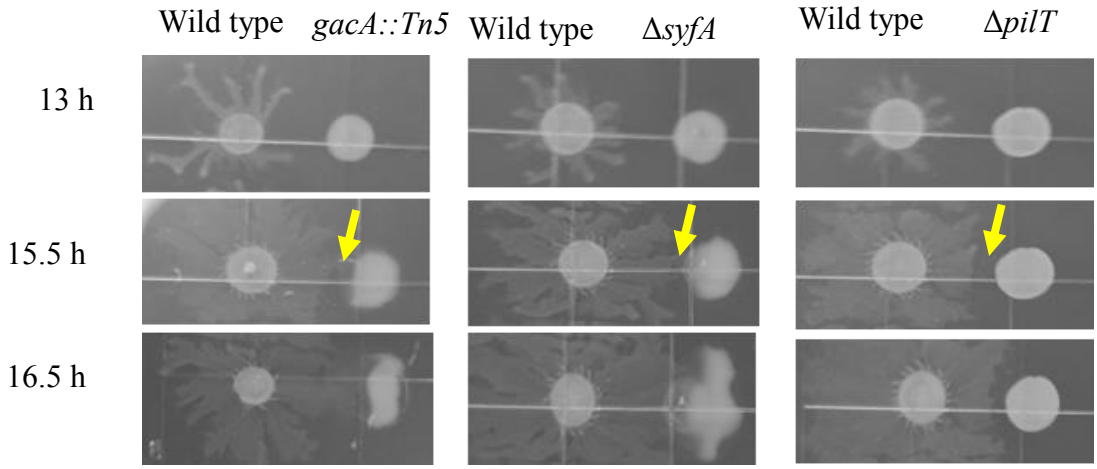

Figure 5. The strains Δ*syfA* and *gacA::Tn5* rapidly move away from the wild type on swarm plates in apparent response to the close proximity of the wild-type tendrils, whereas the strain Δ*pilT* does not.

whole Δ*syfA* and *gacA::Tn5* colonies translocated in the opposite direction as though they were being repelled. In contrast, Δ*pilT* colonies did not translocate when the wild type was in close proximity. Since the Δ*pilT* mutation was phenotypically dominant to the loss of flagellin in Δ*fliC* (Fig. 1), these results do not differentiate between roles for flagella versus type IV pili in this translocation activity, and thus do not provide insight into the roles of type IV pili, flagella or other motility-related cellular components in this movement.

Based on the predicted role of HAA as a repellent (15), we investigated if HAA production was involved in this repelling activity of the wild-type swarm colonies. When

gacA::Tn5 was flanked by the wild type and Δ*rhlA*, it moved toward Δ*rhlA* (Fig. 6A), indicating that loss of HAA resulted in an inability to repel *gacA::Tn5*. We additionally observed that wild-type swarm colony tendrils moved preferentially toward *gacA::Tn5* (see arrows in Fig. 6A),

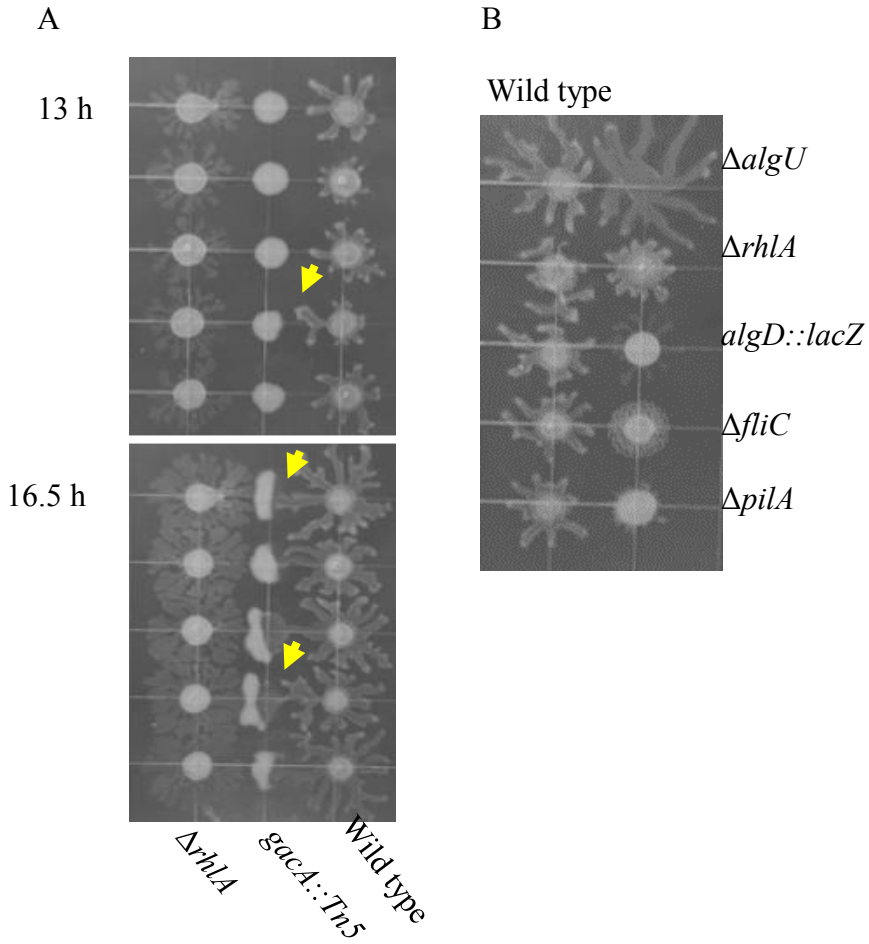

Figure 6. The ability of the wild type to promote translocation of the mutant strains was mutant specific. (A) Colonies of Δ*gacA* preferentially move toward Δ*rhlA* colonies when placed between Δ*rhlA* and wild type swarm colonies and incubated for 13 or 16.5 h. (B) Colonies of mutants that showed motility of any type do not move away from the wild type when placed in close proximity to wild type swarm colonies and incubated for 15.75 h.

causing the wild type to appear as if it was "chasing" *gacA::Tn5*. In contrast, Δ*rhlA* formed tendrils that exhibited symmetrical outward movement (Fig. 6A), indicating that Δ*rhlA* may be deficient in both preferentially swarming toward *gacA::Tn5* and promoting translocation, i.e., repelling it.

To begin to understand the cellular traits involved in translocation in response to an HAA-producing strain, we examined the behavior of a suite of mutants when cultivated adjacent to the wild type on a swarm plate. The wild type promoted translocation of Δ*syfA* and *gacA::Tn5* (Figs. 5 and 6A), but did not promote translocation of Δ*algU,* Δ*algD,* Δ*fliC* or Δ*pilA* (Fig. 6B). Interestingly, Δ*syfA* and *gacA::Tn5* were the only two of these mutants that were completely deficient in motility on swarm media (Figs. 3 and 4), suggesting that the ability to move by any form of motility masked this translocation activity.

Discussion

In this study we demonstrate the role of the type IV pilus, AlgU and alginate in swarming motility and characterize a movement induced in non-swarming mutants by pursuit and production of HAA by cells in close proximity. By evaluating mutants lacking *pilA* and *pilT*, which encode for prepilin and the pilus-retraction protein respectively, we provide the first evidence for the role of the type IV pilus as a contributor to *P. syringae* pv. syringae B728a swarming motility. Additionally, we characterized a unique pattern of surface motility we designate translocation. Furthermore, we demonstrate that AlgU negatively regulates swarming, alginate has a positive impact on swarming motility, and SyfA and GacA are required for swarming motility, while providing quantitative evidence for the role of HAA in tendril formation and repression of swarm colony surface area.

Our evidence indicates not only that flagella and type IV pili are both required for optimal swarming motility in B728a, but also that type IV pili-mediated swarming may be light regulated. A role for the type IV pili in swarming was supported by the reduced swarming of Δ*pilA* when compared to the wild type (Fig. 1). This was further supported by the abolished swarming of Δ*pilT*, although this loss of swarming could be caused by non-retracted pili

interfering with flagellar function. PilT disassembles pilin into subunits, which results in filament retraction. In *P. aeruginosa*, loss of *pilT* results in a hyperpiliated phenotype as type IV pili continue to assemble but are not disassembled (44). We hypothesize that loss of *pilT* in *P. syringae* also results in a hyperpiliated phenotype, and that hyperpiliated cells can no longer rotate their flagella, either because of increased cell-to-cell attachment, mechanical disruption of flagellar rotation, or down-regulation of the genes required for flagellar biosynthesis. Additionally, Δ*fliC*, which was significantly reduced in swarm colony surface area, still exhibited some movement, suggestive of type IV pili-mediated movement, and this movement was abolished in Δ*pilT*Δ*fliC*. This role for a type IV pilus in B728a swarming is consistent with the demonstrated role for type IV pili in the swarming motility of *P. syringae* pv. tabaci 6605 (37, 38) and *P. aeruginosa* (20). The hollow arcs exhibited by Δ*fliC* suggest that the type IV pilus functions in the formation of "rafts" in which cells are packed together along their long axes, a behavior observed microscopically in swarm colonies (2, 20). Consequently, the loss of these "rafts" when Δ*fliC* was incubated in white light (Fig. 1B, C) suggests that light reduces type IV pili formation. Furthermore, neither Δ*pilA* nor Δ*fliC* exhibited the reduced swarming in response to light that was characteristic of the wild type (43), indicating a loss of light-mediated regulation. This is consistent with the possibility that the type IV pilus is subject to regulation by BphP1, a photosensory protein that represses motility in response to red+far-red light (43).

Mutants deficient in swarming that have functional flagella and/or type IV pili move via translocation away from wild-type swarm colonies in close proximity. We observed that *gacA::Tn5* and Δ*syfA*, which do not swarm (Fig. 3, 4) (16, 28), moved away from wild-type swarm colonies in close proximity (Fig. 5). To test if this translocation was possible by all cells that do not move on swarm media, we evaluated Δ*pilT* for translocation away from wild-type

cells in close proximity and found that it did not exhibit this behavior (Fig. 5). Δ*pilT* lacks functional flagella in addition to likely being hyperpiliated, which is predicted to interfere with flagella function, therefore we hypothesize that flagella and/or type IV pili are required for translocation.

Wild-type swarm cells actively pursue non-swarming cells and their repelling abilities may be due to production of HAA. This hypothesis is supported by the preferential translocation of *gacA::Tn5* toward Δ*rhlA* (Fig. 6) when *gacA::Tn5* was inoculated between the wild type and Δ*rhlA* on the same plate, suggesting that cells lacking HAA are incapable of repelling non-swarming cells. HAA acts to modulate tendril organization by functioning as a repellent in *P. aeruginosa* (48) and is predicted to have a similar function in *P. syringae* (15); this is supported by the larger surface area and wider tendrils of Δ*rhlA* (Fig. 4).

Pursuit by the wild type appears to be preferential to cells that do not move on swarm media as wild-type swarm colonies remained symmetrical when in close proximity to mutants that maintained some surface motility (Fig. 6B). The wild type also did not pursue Δ*pilT* (Fig 5), suggesting that it only pursues cells that have the ability to translocate. Pursuit followed by repelling though HAA production may be a mechanism used by B728a to compete with the diverse microbial communities present in the phyllosphere where microbes use a variety of methods to compete for nutrients and space (49). This hypothesis is supported by the high expression of HAA synthesis genes on the leaf surface (21).

Increased salt concentrations repress swarming motility and this repression appears to occur through the sigma factor AlgU independent of its regulation of alginate production. This is supported by the reduced swarming of the wild type on plates amended with increasing concentrations of NaCl (Fig. 2) and the increased swarming of mutants lacking *algU* (Fig. 3). In

contrast, a mutant lacking *algD*, which encodes the protein for the first step of alginate biosynthesis (46), was reduced in swarming motility (Fig. 3), likely due to the ability of alginate to pull water toward swarming cells through its properties as a hygroscopic polymer (27). Taken together, these results suggest that although AlgU induces alginate production (23), which positively contributes to swarming, AlgU negatively regulates swarming through some other pathway. Hyperswarming by an *algU* mutant of *P. fluorescens* was linked to increased flagellar gene expression (50); however, loss of *algU* does not lead to increased flagellar gene expression in *P. syringae* (21).

Collectively, this work further elucidates the variety of factors that contribute to swarming motility and provides the first evidence for a role of type IV pili in swarming motility by *P. syringae* pv. syringae B278a. Furthermore, this work characterizes a novel surface motility that occurs in response to HAA production by B728a swarm colonies. Future work will evaluate the mechanism of light-mediated regulation of type IV pili, test the influence of PilT on flagellar function, explore the role of the type IV pili in translocation by non-swarming mutants, and elucidate the mechanism of AlgU-mediated repression of swarming motility.

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CHAPTER 5. CONCLUSIONS AND FUTURE DIRECTIONS

Bacteria respond to fluctuations in a variety of conditions including temperature, water and nutrient availability, osmotic and oxidative stress, iron and nitrogen limitation, and solar radiation when adapting to the leaf environment. In the last two decades genes encoding photoreceptors have been discovered with increasing frequency in heterotrophic bacteria, including a variety of leaf-associated bacteria and foliar bacterial pathogens, suggesting that light serves as an additional environmental cue to aid in adaptation. Light spectra and intensity may serve as diurnal, seasonal, and positional cues for plant-associated bacteria. Furthermore, periods of intense light exposure are generally correlated with other environmental stresses, including low water availability and high temperature, indicating that light sensing through photoreceptors may be involved in the induction of multiple stress-response pathways. The foliar pathogen *Pseudomonas syringae* is unique among heterotrophic bacteria in encoding two red and/or far-red light-sensing bacteriophytochromes, BphP1 and BphP2, and a blue light-sensing LOV protein, suggesting that light sensing may be particularly important for this organism. In this study we demonstrated that a photoreceptor-mediated signal transduction pathway regulates swarming motility and contributes to plant colonization and virulence by *P. syringae.* Additionally, we demonstrated roles for various cellular traits in swarming motility and characterized a novel coordination of repulsion and induction of surface motility.

We discovered that BphP1 mediates blue-light sensing; however, the mechanism of this sensing has not been elucidated and precedence for blue light-sensing phytochromes in bacteria is limited to a single phytochrome, Cph2 (1). Cph2 encodes three GAF domains, two of which have the capability to interact with distinct chromophores and therefore provide the opportunity for excitation in response to multiple wavelengths (1). In contrast, BphP1 only has one GAF

domain. Although the C-terminal CphP1 GAF domain shows greater absorption in blue light than red light (1), BphP1 encoded by *P. syringae* strain DC3000 showed limited absorption at 470 nm (2), suggesting that increased absorption is not a mechanism of blue-light sensing by BphP1. However, blue-light sensing by BphP1 may be unique to strain B728a. Future work should include evaluating the absorbance spectra of BphP1 from these strains and others, using methods as previously described (2, 3). Additionally, whereas we did not observe autophosphorylation of BphP1 in response to blue light, blue light-mediated autophosphorylation may occur under conditions that were different to those used. pH, temperature, and $MgCl_2$ and KCl concentrations are known to influence protein folding and histidine kinase activity *in vitro*; systematic testing of variations in *in vitro* conditions would enable a more complete examination of potential blue light-mediated autophosphorylation of BphP1.

The mechanism of integration of BphP1 and LOV swarming regulation requires further elucidation, particularly since yeast-two hybrid analysis suggests that LOV and BphP1 do not interact directly (Appendix A). In fact, the results of these analyses suggested that LOV and BphP1 were repelled from one another based on the result that yeast cells expressing both *lov* and *bphP1* exhibited less β-galactosidase activity than the negative control (Appendix A). This result suggests that integration occurs through an alternative component of the LOV/ BphP1-mediated pathway. In this work we demonstrated that Lsr functions downstream of BphP1 to regulate swarming motility, swarming initiation, and lesion development. Additionally, we demonstrate that acyl-homoserine lactone production is modulated by BphP1 to regulate swarming initiation. Future studies should evaluate if direct BphP1-Lsr and LOV-Lsr interactions occur, in addition to determining if BphP1 or LOV interacts with the quorum regulators AefR and AhlR (4). Studies currently underway are utilizing a GFP-based two-hybrid

library to identify additional components involved in light-mediated signaling. qRT-PCR could also be utilized to evaluate if red light or BphP1 regulate quorum molecule production through expression of *ahlI*.

We hypothesize that BphP1-mediated contribution to leaf colonization may be due to BphP1 regulation of aggregate formation. We provided the first evidence for the role of a bacteriophytochrome in plant colonization and demonstrated that BphP1 contributes to initial survival on the leaf surface and negatively regulates colonization in later stages. We hypothesize that the hypermotility of cells lacking *bphP1* results in an inability to form aggregates and thus reduces protection from desiccation stress (5). To test this hypothesis the wild type and Δ*bphOP1* could be tagged with GFP and motile cells inoculated on bean plants for microscopic comparisons of aggregate formation, using methods that were previously described (5). The mechanism of BphP1-mediated regulation of colonization may include components independent of motility based on our demonstration that Lsr does not contribute to colonization. Novel downstream components identified by genome-wide screens performed using a GFP-based two-hybrid system should be tested for a role in leaf colonization.

We demonstrate that light-mediated regulation of swarming motility contributes to *P. syringae* movement in soil and hypothesize that this regulation is a mechanism to avoid stressful conditions during high light exposure. Previous work has suggested that flagellar motility is particularly important for colonization of the rhizosphere (6). Our work shows that type IV pili also contribute to swarming motility. Future work could use the Δ*fliC* and Δ*pilA* mutants to determine if the flagella and pilus contribute equally to motility in the soil. Additionally, mutants lacking *lsr* and *ahlI-ahlR* should be tested to determine if they are required for BphP1-mediated regulation of motility in the soil, as they are not involved in BphP1-mediated contributions to

leaf colonization. Our studies were performed in field conditions so populations were exposed to the complete spectrum of visible light; however, red and far-red light are better able to penetrate soil compared to blue light (7). To confirm that red-light sensing is the primary contributor to light-mediated regulation of motility in the soil despite the potential for integration with blue-light mediated regulation, future studies could compare the motility of the wild type in soils exposed to red+far-red and blue light conditions. We hypothesize that light-mediated repression of swarming motility in the soil likely correlates with diurnal fluctuations in temperature and water availability; however, the mechanisms underlying *P. syringae* adaptation in soil have not been well studied. Bacterial aggregates may protect cells from stressful conditions encountered in the soil similar to their role on leaves (8). GFP-tagged strains could be used to evaluate the role of light-mediated repression of swarming motility in aggregate formation in soil. Additionally, studies in which wild-type bacteria are exposed to various soil water contents and temperatures could be performed to better understand the effect of these conditions on *P. syringae* soil adaptation and motility.

We also hypothesize that light-mediated regulation of swarming has a role in BphP1 and Lsr contributions to lesion development. The increased motility of cells lacking *bphP1* or *lsr* may allow them to influence water efflux from plant cells farther away from the point of inoculation, resulting in larger water-soaked lesions; however, a role for motility in lesion development has not been documented. To evaluate the role of motility in lesion development, the mutants Δ*fliC*, Δ*pilA*, and Δ*fliC*Δ*pilT*, which lack flagella, pili, and surface motility, respectively, can be compared based on the size of the water-soaked lesions they induce. BphP1 and Lsr may also regulate enzymes involved in tissue maceration, which occurs in advanced stages of lesion formation, as mutants lacking *ahlI* and *ahlR* are unable to induce this maceration

(9). Bean pods inoculated with Δ*bphOP1* and Δ*lsr* could be incubated beyond the 48 h used in the current studies to test for a role in tissue maceration.

BphP2 has a domain structure that is unique among bacteriophytochromes; however, the implications of this domain structure in BphP2 function have not been determined. In addition to the photosensory domain and HisKA domains encoded by BphP1, BphP2 has a HWE histidine kinase domain. The other heterotrophic bacteria that are known to encode two bacteriophytochromes, namely *Agrobacterium tumefaciens* and *Pseudomonas putida,* also have one with a HisKA domain, but the second have only HWE domains. We hypothesize that a *bphP1* gene duplication occurred followed by recombination with a gene encoding a HWE domain, and that this unique domain structure resulted in loss of function. This possibility is supported by our inability to detect a role for BphP2 in the analyzed phenotypes. This second histidine kinase domain may also be related to the challenges encountered in purifying BphP2 when expressed in *E. coli* (2) and our associated inability to perform biochemical and protein activity analyses on BphP2. Expressing BphP2 as a truncated protein that only includes the photosensory and HisKA domains may enable purification. Moreover, analysis of autophosphorylation and absorption patterns of this truncated protein could elucidate if the BphP2 originated from a duplication event involving *bphP1*.

Our results demonstrate that the type IV pilus is involved in swarming motility and is light regulated; further experimentation is necessary to determine if BphP1 or LOV are involved in this regulation. A *pilA* deletion mutant was reduced in swarming motility and a *pilT* deletion mutant was completely abolished in swarming motility, demonstrating that the type IV pilus contributes to swarming. However, Δ*pilT*, which is likely hyperpiliated (10), should still have functional flagella. To evaluate if loss of *pilT* results in downregulation of flagella, a flagellar

antigen could be used as a probe of a western blot, as previously described (11). We hypothesize that increased pilus production results in greater cell-to-cell attachment, thus interfering with flagellar function. Our results suggest that type IV pili are regulated by light. To test if BphP1 and LOV are involved in this light regulation, we could construct double mutants such as Δ*pilA*Δ*bphOP1*, Δ*fliC*Δ*bphOP1,* Δ*pilA*Δ*lov*, and Δ*fliC*Δ*lov* and evaluate their swarming motility in white, red, and blue light and dark conditions.

We characterized a novel pattern of surface motility that we designated translocation. Δ*syfA* and *gacA::Tn5* translocated away from wild-type cells in close proximity whereas the Δ*pilT* did not. To evaluate if the type IV pilus or flagellum is required for this type of surface motility, we could construct the double mutants Δ*pilA*Δ*syfA*, Δ*pilAgacA::5,* Δ*fliC*Δ*syfA* and Δ*fliCgacA::5* and test them for translocation. We also provide evidence for the role of 3-(3-hydroxyalkanoyloxy) alkanoic acid (HAA) as a repellent based on that mutants lacking *rhlA* appeared to be unable to induce translocation of Δ*syfA* and *gacA::Tn5* cells in close proximity. HAA is involved in tendril formation through its function as a repellent in *Pseudomonas aeruginosa* (12). Because HAA is present in the culture supernatant, supernatants of the wild type could be evaluated for their ability to induce translocation of Δ*syfA* and *gacA::Tn5*. We have observed that B728a moves toward Δ*syfA* and *gacA::Tn5,* as well as DC3000 (data not shown), suggesting an "active pursuit"; however, the signals that B728a senses and the mechanisms underlying such "active pursuit" behavior are completely unknown. We hypothesize that this pursuit behavior coupled with HAA production to repel another organism may enhance the ability of B728a to compete with other microbes in the phyllosphere. Further studies are required to determine how B728a differentiates between cells it actively pursues and those it does not.

Several lines of evidence illustrate the importance of motility in *P. syringae* leaf colonization and virulence (13, 14). Future studies are required to determine if light-mediated regulation of swarming is the only pathway by which BphP1 contributes to leaf colonization and virulence; only a few of the possible avenues of study are outlined in this chapter. Collectively, this work advances our understanding of the role of light as an important environmental signal for *P. syringae*, the signal transduction pathway associated with BphP1, and the mechanisms of surface motility.

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APPENDIX A. BPHP1 INTERACTS WITH PSYR_2449, PSYR_0886, PSYR_0488, AND PSYR_0489, BUT NOT LOV BASED ON YEAST TWO-HYBRID ANALYSIS

BphP1 and LOV function together to regulate swarming motility (1). To evaluate the mechanism of swarming regulation we sought to identify downstream components involved in the BphP1-LOV signal transduction pathway. Software (2) predicted ten response regulators with a high probability of interacting with BphP1. To evaluate if these predictions were valid we tested for interactions of the histidine kinase domain (HisKA) of BphP1 with the three response regulators with the highest probability of interaction and two other response regulators with lower predicted probability of interaction that appeared to function in a motility related pathway. Yeast cells that expressed *bphP1HisKA* along with either *Psyr_2449* or *Psyr_0489* (Fig. 1) produced significantly more β-galactosidase than the negative control based on visually scoring colony color, suggesting that BphP1 may interact with Psyr_2449 and/or Psyr_0489.

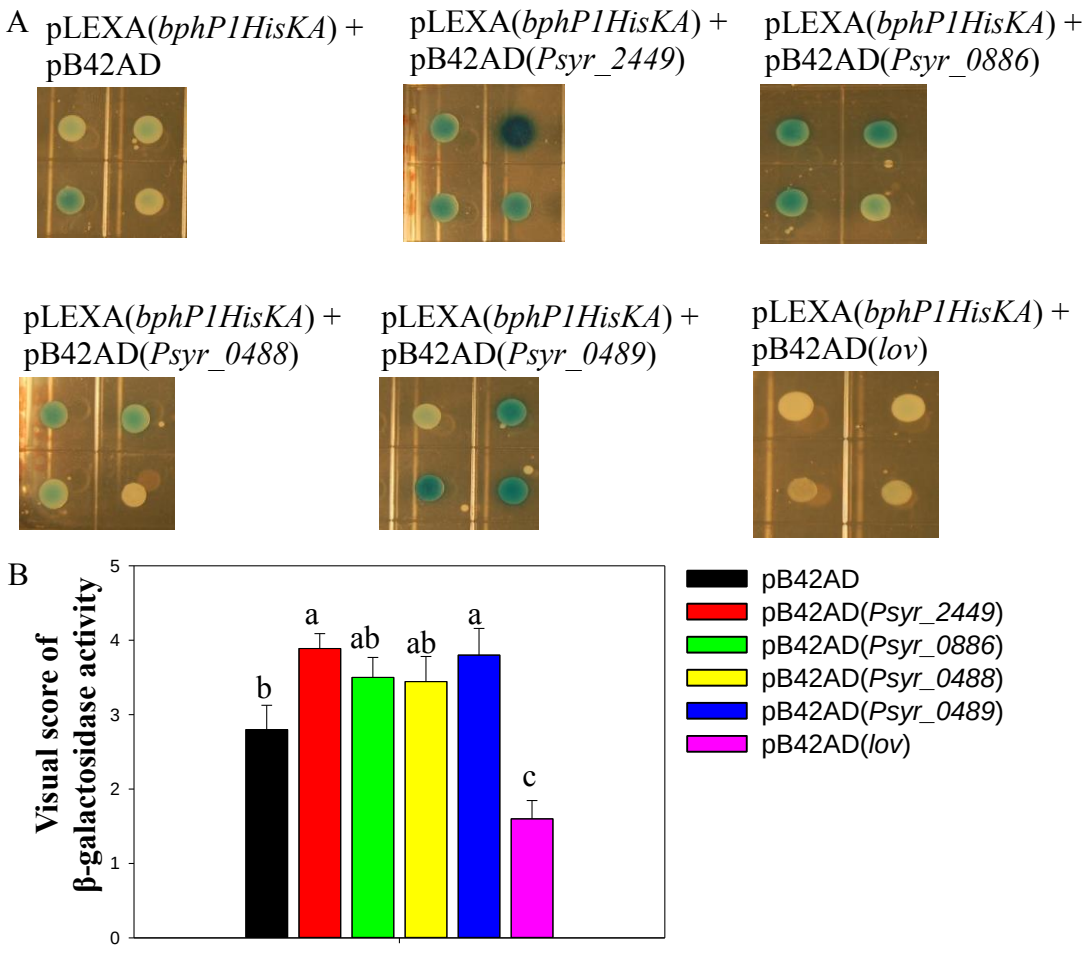

Figure 1. The histidine kinase domain (HisKA) of BphP1 interacts with Psyr_2449, Psyr_0886, Psyr_0488, and Psyr_0489 but not LOV based on β-galactosidase activity when plasmids expressing *bphP1HisKA* fused to the *lacZ* activation domain and *lov* or the potential response regulators fused to the *lacZ* DNA binding domain are introduced via a yeast-two-hybrid system into the same yeast cell. Genes involved in histidine and tryptophan synthesis are encoded on the bait plasmid pLEXA and the prey plasmid pB42AD, respectively, allowing for selection of cells containing both plasmids. pLEXA(*bphP1*HisKA) and pB42AD expressing the potential response regulators or *lov* were introduced into yeast strain EGY48, which lacks the ability to produce histidine and tryptophan, via transformation and were grown on SD medium (6.7 g yeast nitrogen base (Sigma-Aldrich, St. Louis, MO) and 20 g agar/liter) for 3-4 days at 25°C. Ten colonies were selected at random and grown overnight in 1 ml of SD broth. A 10X amino acid mixture containing 300 mg L-isoleucine, 200 mg L-adenine hemisulfate salt, 1000 mg L-leucine, 200 mg L-methionine, 2000 mg L-threonine, 1500 mg L-valine, 200 mg L-arginine, 300 mg L-lysine, 500 mg L-phenylalanine, and 300 mg L-tyrosine per liter was diluted to 1X by adding 65 ml SD medium, 5 ml 20% raffinose, 10 ml 20% galactose, 10 ml BU Salt, 2 g agar, and 100 µl X-gal to make screening plates for colonies that can produce histidine and tryptophan and exhibit β-galactosidase activity. Cells grown in SD medium were concentrated 10-fold by centrifugation, spotted onto screening plates, and grown at 25°C for two days. Following incubation, the ten

spots for each bait-prey construct were photographed, four of which are shown in (A), and were scored on a 1-5 scale for β-galactosidase activity based on the intensity of the blue color (B). Values represent the mean and SE and values indicated by the same letter do not differ significantly ($P<0.05$ comparisons were based on a one-way ANOVA, $n=10$). The plasmid pB42AD(*Psyr_4376*) was constructed and tested but showed inconsistent growth and β-galactosidase activity. Results were repeated three times and the results of a representative experiment are shown in (B).

APPENDIX B. LOV HAS A VARIABLE IMPACT ON *PSEUDOMONAS SYRINGAE* PLANT COLONIZATION

To evaluate the role of LOV in leaf colonization total bacterial populations of the wild type, Δ*lov*, and Δ*lov*(p*LOV*) were tracked overtime on *Phaseolus vulgaris* (cv. Bush Blue Lake 274) leaves following inoculation onto the leaf surface. Due to the previous observation that LOV contributes to swarming motility, Δ*fliC* was included in the first experiment for comparison. Results across the three experiments were variable (Fig. 1), suggesting that LOV may be regulated by a yet unidentified environmental factor that varied across experiments.

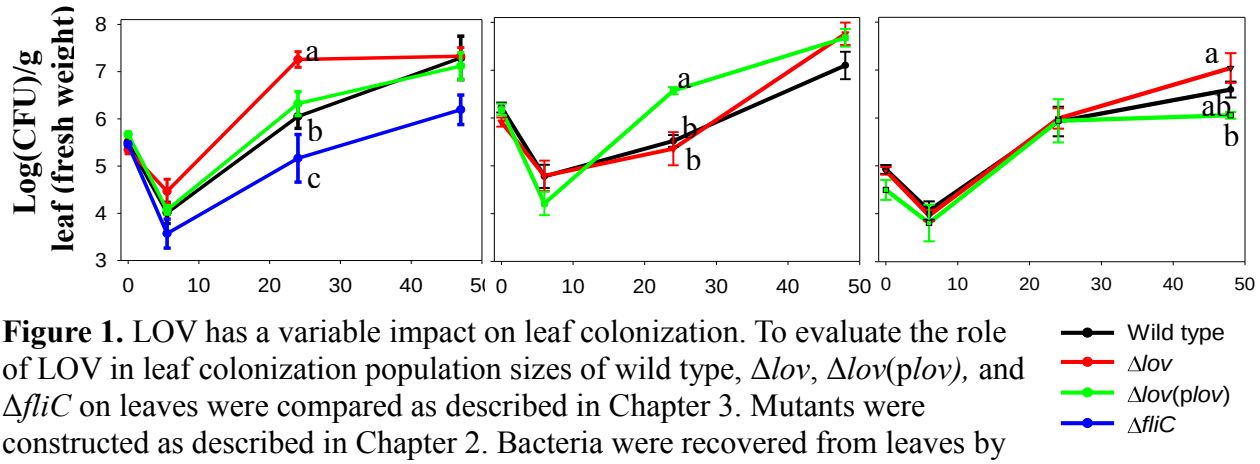

Figure 1. LOV has a variable impact on leaf colonization. To evaluate the role of LOV in leaf colonization population sizes of wild type, Δ*lov*, Δ*lov*(p*lov),* and Δ*fliC* on leaves were compared as described in Chapter 3. Mutants were constructed as described in Chapter 2. Bacteria were recovered from leaves by homogenization of whole leaves. Values represent the mean and SE and values in the same figure indicated by the same letter do not differ significantly for comparisons within a single time point ($P<0.05$ comparisons were based on a one-way ANOVA, n=6).

APPENDIX C. MOTILITY HAS A VARIABLE IMPACT ON THE ABILITY OF *PSEUDOMONAS SYRINGAE* TO GAIN ACCESS TO PROTECTED SITES

BphP1 and LOV were previously demonstrated to regulate swarming motility in *Pseudomonas syring*ae (1). To determine if either BphP1 or LOV contribute to the ability of *P. syringae* to move toward protected sites Δ*bphOP1* and Δ*lov* were inoculated onto leaf surfaces (P. vulgaris cv. Bush Blue Lake 274) and internal populations were determined. In Chapter 4 we demonstrate that type IV pili and flagella also contribute to swarming motility, so Δ*pilT* and Δ*fliC* were also evaluated for movement to protected sites. Results across three experiments were variable (Fig. 1) suggesting that motility has a variable impact on movement to protected sites.

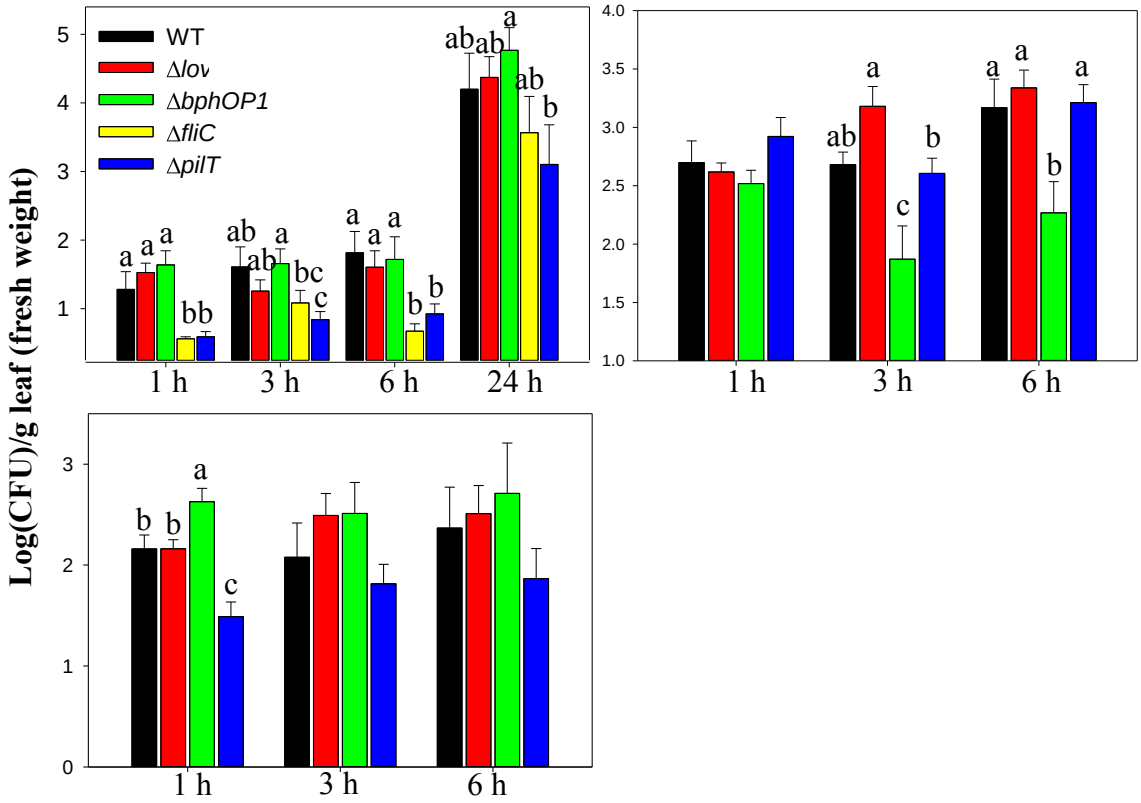

Figure 1. Motility has a variable impact on ability of *P. syringae* to gain access to protected sites. The wild type, Δ*lov*, Δ*bphOP1,* Δ*fliC,* and Δ*pilT* were evaluated for their ability to move to protected sites. Several colonies (3-5) of each strain were recovered from King's B medium containing 1.5% agar following a 48-h incubation and were suspended in 5 ml of phosphate buffer, pH 7 (PB). A suspension of $4x10^6$ cells/ml in PB was inoculated onto the leaves of plants in four pots containing 8-10 two-week old bean plants (*Phaseolus vulgaris* cultivar Bush Blue Lake 274) by application with a hand sprayer. Following inoculation, plants were incubated under white fluorescent bulbs (Ecolux F40C50-Eco, General Electric Co., Fairfield, CT). At each time point two leaves were collected from each pot and the eight leaves representing each strain were incubated in a flask containing 150 ml of 0.02% Tween 20 and 2% sodium hypochlorite with shaking for 3 min. The leaves were then rinsed three times in sterile nanopure water before being transferred into 5 ml of washing buffer (PB with 5 g peptone/l) and homogenized. *P. syringae* populations were enumerated on King's B medium containing Rif and Cyclo. Differences in populations in protected leaf sites among strains at each time point were evaluated by comparing the log-normal values using ANOVA. Values represent mean and SE and values in the same figure indicated by the same letter do not differ significantly for comparisons within a single time point ($P<0.05$ comparisons were based on a one-way ANOVA, n=8).

References

1. Wu L, McGrane RS, & Beattie GA (2013) Light regulation of swarming motility in *Pseudomonas syringae* integrates signaling pathways mediated by a bacteriophytochrome and a LOV Protein. *Mbio* 4(3):e00334-00313.
2. Burger L & van Nimwegen E (2008) Accurate prediction of protein-protein interactions from sequence alignments using a Bayesian method. *Mol. Syst. Biol.* 4.